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SPERMATOGENESIS AND EGG INVESTMENTS IN *LEPTOSYNAPTA CLARKI*,  
*CUCUMARIA LUBRICA* AND *CUCUMARIA PSEUDOCURATA*  
(ECHINODERMATA: HOLOTHUROIDEA), WITH A NOTE ON ACROSOMAL REACTIONS

by



DAVID GRATTAN ATWOOD

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## ABSTRACT

This research has included studies on spermatogenesis, egg investments and acrosomal reactions in the sea cucumbers: *Leptosynapta clarki*, *Cucumaria lubrica* and *Cucumaria pseudocurata*. The most significant findings are listed below.

1) Spermatogonia, spermatocytes and spermatids of *Leptosynapta clarki* and *Cucumaria lubrica*:

Spermatogonia are joined by dense junctions. Proacrosomal granules are evident in late spermatogonia. A tubular body exists in spermatogonia of *C. lubrica*. Spermatocytes are characterized by flagellar formation, centriolar satellites, striated rootlets and a chromatoid body in *L. clarki*. Spermatids are joined by cytoplasmic bridges. The nucleus of the *C. lubrica* spermatid elongates without the aid of microtubules.

2) Spermatozoon of *Leptosynapta clarki*:

The sperm has a circular head measuring  $3.0\ \mu$ , a midpiece containing a mitochondrion and a tail  $45\ \mu$  long. The acrosomal region is at the anterior tip and contains a granule measuring  $0.7\ \mu$  in diameter which consists of dense concentric lamellae. Typical proximal and distal centrioles lie posterior to the nucleus. The distal centriole gives rise to nine satellite projections.

3) Spermatozoon of *Cucumaria lubrica*:

The sperm consists of a cylindrical head,  $1.5\ \mu$  in diameter and  $5.2\ \mu$  in length, a mitochondrial midpiece  $1.9\ \mu$  in length and a tail  $65\ \mu$  long. An acrosomal granule, containing a dense sphere, is located at the anterior tip of the cell. The nucleus is  $6.8\ \mu$  long and tapers to a diameter of  $0.5\ \mu$  at the posterior end. The mitochondrion surrounds the





posterior  $1.6\ \mu$  of the nucleus. Typical proximal and distal centrioles lie posterior to the nucleus.

4) Spermatogonia, spermatocytes and spermatids of *Cucumaria pseudocurata*:

Six stages are evident during spermatogenesis: primary spermatogonia, secondary spermatogonia, early spermatocytes, late spermatocytes, spermatids and spermatozoa. Morphogenesis of the acrosome and striated rootlet is initiated in the early spermatocyte. A morphogenic polarity exists in the developing acrosomal granule. Cell shape is altered in the intermediate spermatid with dorso-ventral compression and anterior-posterior elongation resulting. No microtubules are evident.

5) Spermatozoon of *Cucumaria pseudocurata*:

The tabloid sperm consists of a dorsal surface containing a striated rootlet and a ventral surface containing an acrosome. The head is  $5.5\ \mu$  in length,  $1.2\ \mu$  in width and  $0.8\ \mu$  in depth. A mitochondrion lies at the base of the nucleus. The flagellum,  $70\ \mu$  long, has a 9+3 tubular arrangement in the midtail region. The proximal and distal centrioles contain satellite projections and lie posterior to the nucleus.

6) Acrosomal reaction and egg investments in *Leptosynapta clarki*, *Cucumaria lubrica* and *Cucumaria pseudocurata*:

The sperm of *L. clarki* and *C. lubrica* artificially undergo a typical echinoderm acrosomal reaction, whereas sperm of *C. pseudocurata* do not. Sperm of *C. pseudocurata* attach to the egg surface on their sides rather than head on, where they undergo an atypical acrosomal reaction. The *L. clarki* egg is surrounded by an outer particulate-fibrous layer, follicular cell layer, dense laminate fibrous layer, dense





particulate layer and a lucent particulate layer. In *C. lubrica* a follicular cell layer, dense laminate fibrous layer and dense particulate layer are present. The egg of *C. pseudocurata* is surrounded by a single dense laminate layer.



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## Chapter I

### GENERAL INTRODUCTION





## GENERAL INTRODUCTION

### Fertilization: Historical and Present Perspectives

The fertilization process involves the union of male and female gametes and the combination of genetic material from maternal and paternal sources. Sexual reproduction, through the sorting and shuffling of genes into new and different combinations, provides a constant source of phenotypes for testing against the environment. Even though at any designated time there will be a smaller proportion of well-adapted individuals than would arise from a well-adapted asexual population, a sexually reproducing population has the advantage in its ability to adjust to changing environmental demands.

Echinoderms have been important in fertilization studies due to the fact that they are easily accessible and relatively simple to spawn and fertilize under laboratory conditions. Echinoderm species, besides being tools for reproductive research, are key members of the ecosystems they inhabit owing to their vast numbers and varied interactions with species of other phyla.

Fol (1877), a pioneer in the field of fertilization, was one of the first to describe the penetration of the animal oocyte by a spermatozoon. As a result of studies on fertilization in *Asterias*, Fol (1879) concluded that the directive movement of the sperm through egg investment layers and eventually into the oocyte was the result of an attraction exerted by a cone-like projection put forth from the surface of the egg. Fol suggested that a tenuous filament arose from the egg surface cone, came into contact with approaching spermatozoa and drew the sperm into the



main body of the egg. Similar egg-sperm connecting filaments were observed in holothurians (Iwanzoff, 1898; Hörstadius, 1939b) and hemichordates (Colwin and Colwin, 1949, 1954). Fol (1879) noted that the spermatozoon advanced as the filament progressively shortened until the sperm head reached the cone into which it then entered. The theory that the filament was an outgrowth of the sperm rather than the egg was ruled out at that time since no reduction in volume of the head was observed (Fol, 1879). Subsequent work by Chambers (1923, 1930) on *Asterias* and Hörstadius (1939a) on *Astropecten* substantiated Fol's results. Just (1929) argued that the fertilizing filaments he observed in certain asteroids originated in the head of the spermatozoon rather than on the surface of the egg. It was not until the work of Dan (1952, 1954) on echinoids and Colwin and Colwin (1955) on *Holothuria* and *Asterias* that it was conclusively demonstrated that the sperm head gave rise to an "acrosomal filament" (Dan, 1952) or process which preceded the advancing spermatozoon into the entry cone of the egg at the time of fertilization.

The acrosome was initially termed the "perforatorium" by Waldeyer (1870). Brenda (1887) later determined that this organelle originated from a granule located in a vacuole within the Golgi complex and Lenhossek in 1898 proposed that the term "acrosome" was a more appropriate label. Field in his 1893 study of echinoderm sperm referred to the acrosome as the "centrosome." Because of the close association between the acrosome and the nucleus and the fact that it was situated in a slight nuclear indentation, Field obviously mistook this organelle for the region containing the centrioles.

The elongated acrosomal process consists of a single membrane





surrounding a fibrous (filamentous) shaft. Colwin and Colwin (1963) and Dan et al. (1964) have postulated that the filaments originate through the polymerization of precursor materials present within periacrosomal substances of the acrosome. Tilney et al. (1973) and Jessen et al. (1973) have demonstrated that in *Asterias* (Asteroidea) and *Echinocardium* (Echinoidea) G-actin is evident within the periacrosomal material and that its polymerization into linear filaments, F-actin, is responsible for elongation of the acrosomal process. They propose that these filaments may play a dual role in the fertilization process: 1) extension of the acrosomal apparatus through the egg investment layers and 2) incorporation of the sperm nucleus into the egg.

Popa (1927), working with echinoids, was one of the first to report on the acrosomal process. Since that time much research employing light and electron microscopy has been published on echinoderm species (Dan, 1952, 1954, 1956, 1960, 1967, 1970; Colwin and Colwin, 1955, 1956; Rothschild and Tyler, 1955; Afzelius, 1956; Afzelius and Murray, 1957; Collier, 1959; Haino and Dan, 1961; Bernstein, 1962; Dan et al., 1962, 1964, 1972; Dan and Hagiwara, 1967; Franklin, 1970; Summers and Hylander, 1974).

Prior to electron microscopy there was considerable confusion regarding sperm entry into the egg. Loeb (1917) suggested phagocytosis as a possible mechanism in fertilization, whereby the sperm was engulfed or captured by the oocyte. Lillie (1912) stated that following sperm attachment to the egg membrane in *Nereis*, the cortical cytoplasm of the egg became denser at the point of attachment. Subsequent active streaming of the surrounding cytoplasm carried the sperm into the egg by a centripetal movement. Austin (1951) observed sperm entry into the rat egg with



phase contrast microscopy and concluded that penetration was due to some membrane activity by which the sperm head sank into the vitellus. Following studies on reactive systems of fertilizin-antifertilizin and other surface agents, the concept emerged that sperm attachment was due to specific surface reactions followed by a phagocytotic process which was responsible for the swallowing of the sperm. Tyler (1959, 1960, 1962) elaborated this concept in a hypothesis suggesting "Specific Pinocytosis" or phagocytosis, "as a possible Sperm-Engulfing Process." This implied that the entire sperm cell with its plasma membrane intact was surrounded by a vesicle of egg plasma membrane and transported into the cytoplasm of the oocyte. It was thought that the acrosomal filament or the cytoplasm in which it was anchored played the active role in sperm penetration while the egg plasma membrane was mechanically broken.

Even though these concepts of fertilization were appealing there was no direct evidence to support them. In an electron micrograph of a penetrating sea urchin spermatozoon, Rothschild (1957) showed that the sperm plasma membrane was missing. If the sperm head had penetrated through a process of phagocytosis there should have been at least one membrane surrounding the sperm, namely that of the phagocytotic vesicle. It later became evident from work on *Hydroides* (Annelida) (Colwin and Colwin, 1961) and the rat (Szollosi and Ris, 1961) that sperm penetration actually occurred through a membrane fusion process. Colwin and Colwin (1961) stated: "No matter how the fusion is accomplished, it is clear that by a rather early stage the egg plasma membrane and the sperm plasma membrane become one continuous mosaic membrane, and that the two formerly separate cells then constitute a single cell." Extensive reviews on



fertilization have recently appeared which discuss comparative aspects of this phenomenon throughout the plant and animal kingdoms (Monroy, 1965; Colwin and Colwin, 1967; Austin, 1968; Metz and Monroy, 1969; Longo, 1973).

Summers and Hylander (1974) have suggested that gamete contact (at least in sea urchins) is a two-step process consisting of a binding between extracellular materials on the acrosomal process and the egg plasmalemma and then a membrane fusion between the acrosomal process tip and the oolemma. Aketa (1973) has postulated that a species-specific component is present on the apical end of the echinoid sperm which is complementary to a sperm-binding protein of the egg surface. These two molecules are possibly responsible for both initial species recognition and bonding of the gametes. It has been suggested that such specific sperm molecules are contained within the acrosomal vesicle and are made available to the egg surface following the acrosomal reaction and that these molecules form a structural bond with the vitelline envelope before membrane fusion (Summers and Hylander, 1974).

### Spermatogenesis in Echinoderms: Historical and Present Perspectives

To gain a firm knowledge of the features that enable the egg and sperm to unite, it is essential to understand the structure and development of the spermatozoon. Relatively few studies have been published on spermatogenesis in the phylum Echinodermata. Initial fragmentary reports appeared in the late 1800's on all five classes: holothurians (Jensen, 1883; Field, 1893), crinoids, echinoids, ophiuroids and asteroids (Field, 1893). Since that time the majority of studies have dealt with asteroids (Delavault, 1961; Cognetti and Delavault, 1962; Delavault and Bruslé,





1968; Bruslé, 1968; Smith, 1971) and echinoids (Fuji, 1960; Longo and Anderson, 1969). To date, there has been no detailed fine structural investigation of spermatogonia, spermatocytes and spermatids for any species of the class Holothuroidea.

Field (1893) examined spermatogenesis in 19 species representative of all five echinoderm classes.

Throughout all those different species I have found a very general similarity, though in minor details there is considerable variation. The present preliminary account deals mainly with those general facts, which we have reason to believe obtain throughout the entire class, in those species which have retained the general and typical ontogeny.

. . . by use of new apochromatic homogeneous immersion objectives I have been able to overcome many of the obstacles which have hitherto prevented an exclusive study of the spermatogenesis of the Echinoderms as a group (Field, 1893).

Field found that a general testicular section displayed distinct zones characterized by definite cellular stages: spermatogonia with resting nuclei laid peripherally, next was an area of spermatogonia with nuclei in active mitosis, internal to the spermatogonia was a zone of spermatocytes, then spermatids followed by immature spermatozoa, and finally, in the center of the lumen, mature spermatozoa.

In the asteroid *Leptasterias*, developing male germinal cells form fingers which reach out from the germinal epithelium into the testicular lumen (Smith, 1971). The cells of the fingers are layered into zones according to maturity from spermatogonia to mature spermatozoa. In the echinoids *Arbacia* and *Strongylocentrotus* (Longo and Anderson, 1969) the germinal cells are arranged in a series of cell types progressing from spermatogonia (normally in contact with the testicular wall) to spermatocytes (located among and internal to the spermatogonia) to spermatids and spermatozoa (occurring centrally in the lumen). Developing germinal



cells are not divided into distinct zones but are generally more mature as they proceed from the gonadal wall toward the lumen.

Jensen (1883) noted several basic characteristics of the spermatogonia, spermatocytes and maturing spermatozoa in the holothurian *Cucumaria frondosa*. The spermatogonia contain a large nucleus in relation to the surrounding homogeneous cytoplasm. Lipid droplets of varying sizes are observed surrounding the homogeneous nucleus. The nucleolus, not visible in unstained preparations, is very distinctive when properly stained. Spermatogonia give rise to spermatocytes which contain lipid droplets and consist of a smaller volume than noted in spermatogonia. Following formation of the tail, in maturing sperm, cytoplasm condenses and becomes progressively smaller. The tail does not appear to develop from a predetermined point within the cellular protoplasm. At this stage, lipid droplets are more numerous, the nucleus decreases in size and the nucleolus is no longer visible.

Field (1893) observed in various echinoderm species that a great number of darkly stained granules appeared in the spermatid stage and gradually fused into larger and larger refringent bodies, which in the mature sperm existed as a single large Nebenkern (mitochondrion). In the late spermatid the Nebenkern could take any position in the cytoplasm with reference to the nucleus, but in the mature sperm was always posterior to the nucleus. It was suggested that this change in position was due to mechanical causes, that is, the Nebenkern was drawn into its final location by the changes of the cytoplasm of the spermatid into the tail of the spermatozoon, and the pressure from the cell membrane of the spermatid which became tightly drawn over the head of the mature sperm.



Longo and Anderson (1969) have demonstrated many of the finer points of spermiogenesis in the echinoids *Arbacia* and *Strongylocentrotus*. Echinoid spermatids are connected by short intercellular bridges which are maintained until the sperm cells are nearly mature. Morphogenesis of the membrane-bound acrosomal granule is initiated in the spermatid stage and is associated with the Golgi complex. Early spermatids, which are irregularly circular, elongate during the later stages of spermiogenesis as nuclear chromatin material condenses. The spermatid develops an anterior depression which houses the acrosomal granule and a posterior indentation termed the centriolar fossa. They assume that the single large mitochondrion, which lies posterior to the nucleus, develops through a fusion of smaller ovoid mitochondria noted in earlier stages. Proximal and distal centrioles occur in the midpiece region posterior to the centriolar fossa.

#### Spermatozoan Structure in Echinoderms: Historical and Present Perspectives

The spermatozoon is a highly specialized cell with a few very precise functions, namely, to transport the genetic material to the oocyte and to introduce the material into the oocyte. Comparative spermatologists have suggested that sperm structure is better correlated with the nature of the environment in which fertilization occurs than with phylogeny (Franzén, 1970; Afzelius, 1972). Spermatozoa which are released freely into the water and fertilize externally have been termed "primitive" (Franzén, 1970). The primitive spermatozoon normally contains a rounded or conical nucleus posterior to a small apical acrosome, a short midpiece consisting of one or a few mitochondria arranged around





a proximal centriole and basal body, and a single flagellum displaying a typical 9+2 tubular pattern (Franzén, 1970). Such sperm are found throughout the animal kingdom occurring in the following phyla: Porifera, Cnidaria, Ctenophora, Nemertini, Ashelminthes, Annelida, Mollusca, Arthropoda, Brachiopoda, Sipunculida, Echiurida, Echinodermata and Chordata (Franzén, 1970; Afzelius, 1972).

Since the late 19th century, numerous light microscopic observations have dealt with sperm morphology in the phylum Echinodermata (Field, 1893, 1895; Retzius, 1905, 1910; Dan, 1950, 1952, 1954; Rothschild, 1951; Colwin and Colwin, 1955, 1956; Chia and Buchanan, 1969). More recently, various detailed anatomical accounts employing electron microscopy have appeared (Afzelius, 1955, 1959, 1972; Dan, 1960, 1967, 1970; Bernstein, 1962; Dan et al., 1964; Franklin, 1965, 1970; Hagiwara et al., 1967; Anderson, 1968; Austin, 1968; Longo and Anderson, 1969; Fawcett, 1970; Inoue et al., 1970; Dan and Sirakami, 1971; Summers, 1972; Longo, 1973; Marshall and Luykx, 1973; Summers and Hylander, 1974; Chia et al., in press; Fontaine and Lambert, unpublished manuscript). The majority of these fine structural studies (usually concerned with the morphology and reactivity of the acrosomal region) have dealt with the classes Echinoidea and Asteroidea. Fragmentary studies have been reported in the classes Ophiuroidea (Dan, 1967, 1970), Crinoidea (Dan, 1967, 1970) and Holothuroidea (Dan, 1967; Summers, 1972). To date, no detailed published account is available concerning the ultrastructure of the spermatozoon from any species of the class Holothuroidea.

Field (1893) studied spermatozoa from the five echinoderm classes and reported the following rather accurate observations:



The spermatozoon must be studied alive. It is so extremely delicate that the greatest care must be exercised in technique. Satisfactory permanent preparations are scarcely to be hoped for. No one method should be relied upon. The results which I have obtained from living cells stained upon the slide, the changes being watched under the microscope, have been confirmed by killed and hardened material. For the latter, I have teased the testes, in various conditions of advancement towards maturity, in a very small quantity of sea water: then Flemming's Chrom-osm-acetic, strong formula; or Platinum chloride, 0,3% for 24 hours, or more: wash in water for 24 hours; stain either in safranin, gentian violet (decolorize in water slightly acidulated), or in Delafield's Haematoxylin; mount in glycerine; dissociate cells by tapping coverglass gently (Field, 1893).

The nucleus varies greatly in size and shape in the different groups; however, in crinoids and echinoids it is generally conical and small, whereas in holothurians, asteroids and ophiuroids it is spherical and generally larger than in the echinoids. At the apical end of the nucleus a cup-shaped depression containing a highly refringent spherical body, the "centrosome" is noted. The size of the centrosome varies greatly in the different groups, being relatively small in echinoids ( $0.3-0.66 \mu$ ) and comparatively large in holothurians, asteroids and ophiuroids ( $1.3 \mu$ ). This spherical body fits tightly into the anterior depression and can be mechanically separated from the nucleus. In the asteroids investigated, the centrosome appears to consist of two parts: a clearer, slightly staining refringent material, spherical in shape, surrounding a deeply staining dumbbell-shaped body. "This latter reminds one strongly of the figures given by various investigators for the first stage of the division of the centrosome" (Field, 1893). Posterior to the nucleus is a Nebenkern, next in size to the nucleus and flattened in the anterior-posterior plane. This structure varies in size in different species and contains granules of various sizes. The tail of the sperm forms from the cytoplasm of the spermatid and is united with the



cell membrane rather than attached to the nucleus (Field, 1893).

There have been at least 101 echinoderm species for which basic sperm morphology has been reported. Of this total number, 37 are echinoids, 23 asteroids, 23 holothurians, 11 ophiuroids and 7 crinoids (Chia et al., in press). All of these sperm typify the primitive type sperm and contain either a spherical (Asteroidea, Crinoidea, Ophiuroidea, Holothuroidea) or conical (Echinoidea) shaped head positioned anteriorly to a short midpiece consisting of a single uniformly shaped mitochondrion surrounding the centriolar region. A prominent acrosome always occurs at the apex of the nucleus. Except for slight modifications, all sperm of a particular class of echinoderms are morphologically similar and, therefore, the general theory has arisen that sperm structure remains the same within each of the five echinoderm classes.

The outstanding variation in sperm structure among the majority of primitive sperm is in the size and organization of the acrosomal region (Fawcett, 1970; Franklin, 1970). With few exceptions the basic structural plan is retained in all primitive, external fertilizing spermatozoa investigated. In situations where unique environmental demands in sperm transport from the male to the egg have arisen, structural modifications have evolved (Franzén, 1970; Afzelius, 1972). The extent that a spermatozoon deviates from the primitive type is determined by the extensiveness of the external demands placed upon the biology of propagation of that species. Spermatogenesis, likewise, can be correlated with the biology of propagation. By studying the various stages of cytogenesis of the sperm it can be determined at which point, if any, the cell begins to deviate from its primitive features. Franzén (1956)





argues that obvious differences in spermiogenesis between two organisms or groups of organisms may have phylogenetic as well as biological consequences.

### Thesis Research Problem

Holothurian species inhabit all seas at all depths with many ecological and anatomical questions being unanswered.

Doubtless there yet remain many undiscovered species of Holothuriadae in the British seas. Of Starfishes we must not expect to find many more kinds, though *Goniaster miliaris*, and some few others which have been seen on the Norwegian shores, may be looked for. Of Sea-Urchins there are probably still fewer unnoticed; but of the Sea-Cucumbers many. Their comparatively unattractive aspect, the difficulty of preserving them (they must always be kept in spirits), their habitat in the sea, and the little attention that has hitherto been paid to them by native zoologists, all lead me to believe that many species have been passed over. Much yet remains to be done towards a full investigation of the anatomy of the Sea-Cucumbers, more especially with a view to a comparison of the structure of the Molluscan with the Annelidous forms of Holothuriadae (Forbes, 1841).

While examining spermatozoa from various holothurian species with light microscopy, it was noted that extensive morphological variation exists in the class. The basic holothurian sperm shape (spherical) is typified by *Leptosynapta clarki*, whereas the shape in *Cucumaria lubrica* is cylindrical (torpedo-shaped) and that in *Cucumaria pseudocurata* is tabloid (elongated and compressed). Closer examination with the light microscope revealed that the *C. pseudocurata* spermatozoon had definite ventral and dorsal surfaces, an extensive groove on the dorsal side and an acrosome-like structure on the ventral side. It is logical to assume that the process of fertilization in *L. clarki* and *C. lubrica* is very similar to that described for other echinoderm species. Likewise,



it can be theorized that the extensively modified sperm morphology in *C. pseudocurata* corresponds to a modified process of fertilization. To understand completely the process of fertilization in a particular species it is first necessary to investigate the process of spermatogenesis, biology of propagation (packaging of sperm for transport to the egg, medium in which sperm must locomote to fertilize the egg, location of egg when fertilized), acrosomal reaction and egg investment substructure.

The purpose of the present study is to investigate spermatogenesis, to a limited extent the biology of propagation, acrosomal reaction and egg investment substructure in three species of holothurians which live under different environmental conditions and exhibit different reproductive habits. Hopefully, new principles will come to light which will add to the existing knowledge of the process of fertilization and spermatozoan structure in marine invertebrates. *Leptosynapta clarki* (Heding, 1928) is an ovarian brooder with internal fertilization which lives in an intertidal sandy habitat. *Cucumaria lubrica* (Clark, 1901) is an external ventral surface brooder with external fertilization which occurs on subtidal rock surfaces in swift water currents. *Cucumaria pseudocurata* (Deichmann, 1938), also an external ventral surface brooder, occurs intertidally within mussel beds attached to rock surfaces.

The thesis research was directed towards answering the following specific questions:

- 1) How is spermatogenesis in the class Holothuroidea comparable to that reported in other echinoderm classes; and is there variation in the spermatogenic process within the holothurian class?



- 2) Do holothurian spermatozoa conform to the primitive sperm type exhibited by other echinoderm species; and if not, then at which stages during spermatogenesis are modifications introduced and how are these alterations reflected in the spermatozoa?
- 3) Does spermatozoan morphology differ significantly within the holothurians; and if so, is it possible to correlate changes in spermatozoan morphology with an altered biology of propagation (sperm transport from the male to the egg) or an alternative factor such as egg investments?



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## Chapter II

SPERMATOGONIA, SPERMATOCYTES AND SPERMATIDS OF  
*LEPTOSYNAPTA CLARKI* AND *CUCUMARIA LUBRICA*



# Fine structure of spermatogonia, spermatocytes, and spermatids of the sea cucumbers *Cucumaria lubrica* and *Leptosynapta clarki* (Echinodermata: Holothuroidea)

DAVID G. ATWOOD

Department of Zoology, University of Alberta, Edmonton, Alberta, T6G 2E1

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Spermatogonia, which usually lie in close contact with the basal lamina of the testicular wall, and are joined together by desmosomes, contain a roughly circular nucleus with one or two nucleoli. Small proacrosomal granules are first evident in late spermatogonia. A tubular body ( $0.2 \times 1.5$  microns ( $\mu$ )) consisting of a parallel array of tubules is observed in spermatogonia of *Cucumaria*. Spermatocytes were identified by the presence of flagellar formation, large proacrosomal granules, and centriolar satellite projections. Striated rootlets of the two species differ morphologically. A dense chromatoid body is present in spermatocytes of *Leptosynapta*. Spermatids of both species are joined by cytoplasmic bridges. By late spermiogenesis, the nucleus of *Leptosynapta* is roughly circular, whereas that of *Cucumaria* is tapered at both ends. No microtubules were observed during the elongation of the *Cucumaria* nucleus. Fibrous projections extend from the proximal centriole of both species into the centriolar fossa. It is suggested that only one mitochondrion is present throughout spermatogenesis. This implies that the mitochondrion transforms from a highly branched tubular structure to a simple compact organelle. Morphogenesis of the acrosome is described.

ATWOOD, D. G. 1974. Fine structure of spermatogonia, spermatocytes, and spermatids of the sea cucumbers *Cucumaria lubrica* and *Leptosynapta clarki* (Echinodermata: Holothuroidea). Can. J. Zool. 52: 1389-1396.

Les spermatogonies, ordinairement situées très près de la membrane basale de la paroi testiculaire et reliées par des desmosomes, contiennent au noyau à peu près circulaire à un ou deux nucléoles. Ce n'est que dans les spermatogonies avancées que l'on observe des petits granules proacrosomiques. On peut voir, dans les spermatogonies de *Cucumaria*, un corps tubulaire ( $0.2 \times 1.5$  microns ( $\mu$ )) constitué de tubules disposés parallèlement. Les spermatocytes se reconnaissent à la présence d'une formation flagellaire, de gros granules proacrosomiques et de projections satellites autour du centriole. La morphologie des radicules striées n'est pas la même chez les deux espèces. Il y a, dans les spermatocytes de *Leptosynapta* un corps chromatoid dense. Les spermatides des deux espèces sont reliées par des ponts cytoplasmiques. Vers la fin de la spermiogénèse, le noyau de *Leptosynapta* est à peu près circulaire, alors que celui de *Cucumaria* est effilé aux deux extrémités. On ne voit pas de microtubules durant l'élongation du noyau chez *Cucumaria*. Chez les deux espèces, des projections fibreuses sortent du centriole proximal pour s'étendre dans la fosse du centriole. On croit que, durant toute la spermatogénèse, une seule mitochondrie est présente. La mitochondrie se formerait alors à partir d'une structure tubulaire très ramifiée qui se transformerait en organe compact unique. On décrit ici la morphogénèse de l'acrosome. [Traduit par le journal]

## Introduction

Relatively few studies have been published on sperm differentiation in the phylum Echinodermata (Delavault 1961; Delavault and Bruslé 1968; Bruslé 1968; Longo and Anderson 1969; Smith 1971). The fine structure of the spermatozoa has been described for several species of holothurians (Summers *et al.* 1971; Summers 1972; Atwood 1974; Atwood and Chia 1974; A. Fontaine and P. Lambert, unpublished). However, there has been no fine-structural investigation of the male germinal cells less mature than spermatozoa for any holothurian. Therefore, the purpose of the present study is to

describe the fine structure of the spermatogonia, spermatocytes, and spermatids of two species of sea cucumbers.

*Cucumaria lubrica*, an external brooder, belongs to the order Dendrochirotia. The mature male contains a gonad consisting of numerous unbranched tubules opening into a common gonoduct leading to the gonopore. The spermatozoon consists of a cylindrical (torpedo-shaped) head bounded posteriorly by a large, single mitochondrial mass (Atwood and Chia 1974). *Leptosynapta clarki* is an ovarian brooding sea cucumber which belongs to the order Apoda. Males contain a gonad of two elongate, slender



tubules which branch dichotomously many times. The two main testicular tubules open into a short gonoduct, connected to the anterior region of the dorsal mesentery, which passes to an inconspicuous gonopore. Mature sperm contain a circular head anterior to a single mitochondrion (Atwood 1974). The major morphological differences in sperm structure occur in the acrosome, nucleus, and positioning of the mitochondrion.

### Materials and Methods

*Cucumaria lubrica* were collected subtidally from August to December, 1973, at Eagle Point, and *Leptosynapta clarki* from the sandy substratum of False Bay from September to December, 1973, San Juan Island, Washington. Testes were removed from mature males and fixed in a glutaraldehyde-H<sub>2</sub>O<sub>2</sub> mixture prepared as follows. Twenty-five percent glutaraldehyde (Fisher Scientific Company) is diluted to a 2.5% solution buffered to pH 7.6 with 0.34 M sodium chloride and 0.4 M phosphate buffer at room temperature. Thirty percent H<sub>2</sub>O<sub>2</sub> (Fisher Scientific Company) is added to 50 ml of the fixative, with continuous stirring, in the amount of 10 drops. Fixation was for 2½ h at room temperature. A similar glutaraldehyde-H<sub>2</sub>O<sub>2</sub> technique has been reported by Peracchia and Mittler (1972). Tissues were then passed into 2.5% glutaraldehyde (with NaCl and buffer at pH 7.6) for 1½ h at room temperature, washed for 1 h in buffer, and post-fixed in 2% osmium tetroxide (in 0.4 M phosphate buffer) for 2 h at room temperature.

Testicular tissues were then rinsed in 0.05 M maleic acid (pH 5.2) for 30 min with three changes and stained en bloc in saturated aqueous uranyl acetate for 30 min. Specimens were again rinsed in 0.05 M maleic acid, dehydrated, embedded in Araldite 502, and sectioned with a Porter-Blum-MT-2 ultramicrotome. Sections from 20 animals were stained with saturated aqueous uranyl acetate and 0.2% lead citrate and observed with a Philips EM 200. For light microscopy, Araldite sections were cut at 1 µ and stained according to Richardson *et al.* (1960).

### Observations

Since the fine structure of spermatogenesis in *Cucumaria* and *Leptosynapta* is basically similar, the following observations and discussion pertain to both species. Ultrastructural differences between the two species will be noted where appropriate.

#### Spermatogonia

Spermatogonial cells of *Cucumaria* and *Leptosynapta* occur individually, not connected by cytoplasmic bridges. Spermatogonial intercellular connections have, however, been reported in a wide variety of organisms (Moens and Go

1972; Oliver and Brinton 1972; Franc 1973). Spermatogonia are not organized into a definitive layer segregated from developing spermatocytes and spermatids as evidenced in various other animals (Fig. 1) (Smith 1971; Moore and Dixon 1972). Generally, the spermatogonia lie in close contact with the basal lamina of the testicular wall (Fig. 2) but are frequently scattered among spermatocytes in the lumen of the gonad (Atwood 1973). Spermatogonia of *Leptosynapta* appear to be more erratic in location than those of *Cucumaria*. The cells, about 7.5 µ in diameter, contain a large, roughly circular nucleus, measuring 5.5 µ in diameter (Fig. 2), with no indentations. Spermatogonial nuclear indentations have been shown in other organisms (Reed and Stanley 1972; Nagy and Edmonds 1973). The nucleoplasm consists of a fine homogeneous matrix in which is suspended condensed chromatin distributed around the periphery of the nucleus and widely scattered throughout central regions (Fig. 2). Peripheral chromatin is interrupted at close intervals opposite nuclear pores. Spermatogonia, joined together by desmosome-like structures (Fig. 5), normally contain two nucleoli in *Cucumaria* (Fig. 4) and one in *Leptosynapta*.

Cytoplasm is extensive and contains a Golgi complex in close proximity to the nuclear membrane as well as numerous tubular and ovoid mitochondria (Fig. 3). A single Golgi complex is present as in spermatogonia of echinoids (Longo and Anderson 1969), whereas multiple complexes have been reported in other phyla (Stagni and Lucchi 1970; McLaren 1973). Each spermatogonium contains several large membrane-bound electron-dense granules measuring from 0.3 to 0.8 µ in diameter (Fig. 3) (McLaren 1973). Present also in the cytoplasm is occasional agranular endoplasmic reticulum, very limited granular endoplasmic reticulum, numerous free ribosomes (Fig. 4), and few polysomes. Multivesicular structures varying in size and cellular location (Fig. 5), as well as large lipid droplets varying in number from one to five per cell (Figs. 5, 7), were observed in most cells. Two centrioles, slightly angular to each other (Fig. 6), lie in the peripheral cytoplasm commonly associated with the Golgi complex. It is believed that morphogenesis of the flagellum and associated projections of the distal centriole have not yet been initiated at the spermatogonial stage.





Occasional spermatogonia (and rarely early spermatocytes) of *Cucumaria* contain a tubular body measuring about  $0.2 \times 1.5 \mu$  that consists of parallel microtubules (Fig. 7). Each tubule is about  $10 \mu$  in diameter and is separated from adjacent tubules by a distance of  $20 \mu$ . Electron-dense particles measuring about  $10 \mu$  in diameter occur in rows among the tubules. Such bodies have never been observed in *Leptosynapta*. A similar tubular structure has previously been reported in human (Nagano 1969; Rowley *et al.* 1971) and rooster (Nagano 1969) spermatogonia and referred to as the crystalloid of Lubarsch (Lubarsch 1896). The cytoplasmic structure observed in humans appears, however, to be somewhat larger, measuring  $0.5 \times 2.0 \mu$ , and contains rows of electron-dense particles with a slightly greater diameter ( $15 \mu$ ) than those reported in *Cucumaria* ( $10 \mu$ ) (Rowley *et al.* 1971). The function of this organelle remains obscure.

Both *Cucumaria* and *Leptosynapta* spermatogonia contain numerous cytoplasmic microtubules lying in close proximity to the centrioles and Golgi complex. In late spermatogonia, occasional small membrane-bound proacrosomal vesicles can be observed in the general area of the centrioles (Fig. 6). Evidently, acrosomal formation is initiated in the late spermatogonial stage.

### Spermatocytes

Early spermatocytes (preleptotene and leptotene stages) have an average diameter of  $6 \mu$ , with a nucleus measuring  $5 \mu$  in diameter containing a single nucleolus. Late spermatocytes (zygotene, pachytene, and diplotene stages) were easily identified by the presence of synaptonemal complexes (Fig. 8) (King and Akai 1971; Esponda and Stockert 1972; Reed and Stanley 1972). These tripartite structures consist of two lateral elements measuring about  $300 \text{ \AA}$  wide and a central element about  $150 \text{ \AA}$  wide. Fine filaments travel from the central element through electron-light spaces to the lateral elements. Wettstein and Sotelo (1971) have shown that the central element varies in substructure in insects and can be correlated with major taxonomic categories. The substructure of the median element has not been resolved in *Cucumaria* and *Leptosynapta*. The late spermatocyte (about  $5.5 \mu$  in diameter) contains a nucleus measuring  $4.5 \mu$

and was never observed to contain nucleoli. Spermatocytes and early spermatids of various other organisms have been shown to contain nucleoli (Schin 1965; Moore and Dixon 1972).

Free ribosomes, few polysomes, and limited endoplasmic reticulum, predominantly agranular, are present in the cytoplasm. Agranular endoplasmic reticulum becomes more abundant in this stage than in spermatogonia. The two centrioles (proximal and distal), lying perpendicular to one another, occur in the peripheral zone of the cell. Extending from the distal centriole is a flagellum, which has a typical  $9 + 2$  tubular configuration (Fig. 9). Nine centriolar satellite projections extend out from the tubular array of the distal centriole. Each projection consists of a primary branch, two secondary branches, and a network of tertiary branches (Figs. 10, 13). Both centrioles lie in close proximity to the Golgi complex (Fig. 9) and are in intimate association with numerous microtubules. In *Cucumaria*, extending from the centriolar satellite region past the proximal centriole is a striated fibrous rootlet having an axial periodicity of  $62 \mu$  (Fig. 9). Elements of the striated rootlet appear to be in contact with the proximal surface of the distal centriole as well as the proximal centriole (Fig. 9).

Striated rootlet-like structures were observed very infrequently in *Leptosynapta* spermatocytes and seem to differ morphologically from those of *Cucumaria*. The rootlet, which occurs either singly or as a bundle of filaments, exhibits a periodicity of  $43 \mu$ , and when single appears to bifurcate as it passes toward the satellite elements of the distal centriole (Fig. 13). No contact was ever observed between the elements of the rootlet and the proximal centriole. Similar bundles of cross-striated filaments have been reported in spermatocytes of *Xenopus* (Reed and Stanley 1972).

Formation of the acrosomal components by the Golgi complex is accelerated in the spermatocyte stage in both *Cucumaria* and *Leptosynapta*. In close association with the cisternae of the complex are small membrane-bound proacrosomal vesicles of varying sizes. Many appear to be void of contents, whereas others contain electron-dense materials (Fig. 11). At the top of Fig. 11 can be seen large proacrosomal granules that are presumably the result of the fusion of smaller vesicles. The assembly of a daughter



centriole in the spermatocyte stage is shown in Fig. 12. The immature daughter centriole (pro-centriole) is positioned in close proximity to the mother and lies at an angle of less than  $90^\circ$ . The pro-centriole is being assembled at the base of the mother in close association with the Golgi complex (Fig. 12). In view of the less than  $90^\circ$  angle between centrioles, and the data published by Friedlander and Wahrman (1966) on a neuropteran insect, it can be ventured that this spermatocyte is in the pachytene – early diplotene stage of meiosis.

A densely staining chromatoid body is present in the cytoplasm of *Leptosynapta* spermatocytes (Fig. 14). This cytoplasmic structure, which is frequently surrounded by mitochondria, normally lies closely associated with the nuclear envelope (Fig. 14). On several occasions the chromatoid body was observed in the general area of the distal centriole. This honeycomb-shaped structure measures about  $0.8 \mu$  in diameter and has a

fine granular consistency. Multiple bodies have never been observed within a single spermatocyte. No such cytoplasmic inclusion has been noted in spermatocytes of *Cucumaria*. A similar chromatoid body has been described in various vertebrate and invertebrate organisms (Sud 1961a; 1961b; Fawcett *et al.* 1970; Comings and Okada 1972). Previously, all invertebrate occurrences have been limited to the classes Arachnida, Crustacea, and Insecta of the phylum Arthropoda.

### Spermatids

Early spermatids measure about  $3.5 \mu$  in diameter and contain an irregularly circular nucleus ( $3 \mu$  in diameter) with chromatin of a patchy appearance. During the process of spermiogenesis the nucleus becomes relatively circular, with the chromatin condensing to a state of coarse granules interconnected by fine fibrous materials (Fig. 15). The chromatin then

Fig. 1. Cross-sectional light micrograph of a portion of the testes. S, spermatozoa; SC, spermatocytes; SG, spermatogonia; SP, spermatids. *Cucumaria*, 550  $\times$ . Fig. 2. Spermatogonium in close association with the testicular wall. BL, basal lamina of the testicular wall; N, nucleus; arrow, nucleolus. *Cucumaria*, 9500  $\times$ . Fig. 3. Spermatogonium of *Cucumaria*. DG, electron-dense granules; M, mitochondria. 27 000  $\times$ . Fig. 4. Spermatogonium with nucleus containing two nucleoli. R, free ribosomes. *Cucumaria*, 45 000  $\times$ . Fig. 5. Cytoplasm of a *Cucumaria* spermatogonium. L, lipid droplets; V, multivesicular body; arrow, desmosome-like structures. 19 000  $\times$ . Fig. 6. Cytoplasmic region of *Leptosynapta* spermatogonium. C, two centrioles lying almost perpendicular to each other; P, small membrane-bound proacrosomal granules. 32 500  $\times$ . Fig. 7. Tubular body in the cytoplasm of *Cucumaria* spermatogonium. TB, tubular body; L, lipid droplet. 49 000  $\times$ . Fig. 8. Electron micrograph showing synaptonemal complexes in the nuclear region of a spermatocyte. Arrows, synaptonemal complexes. *Cucumaria*, 13 500  $\times$ .

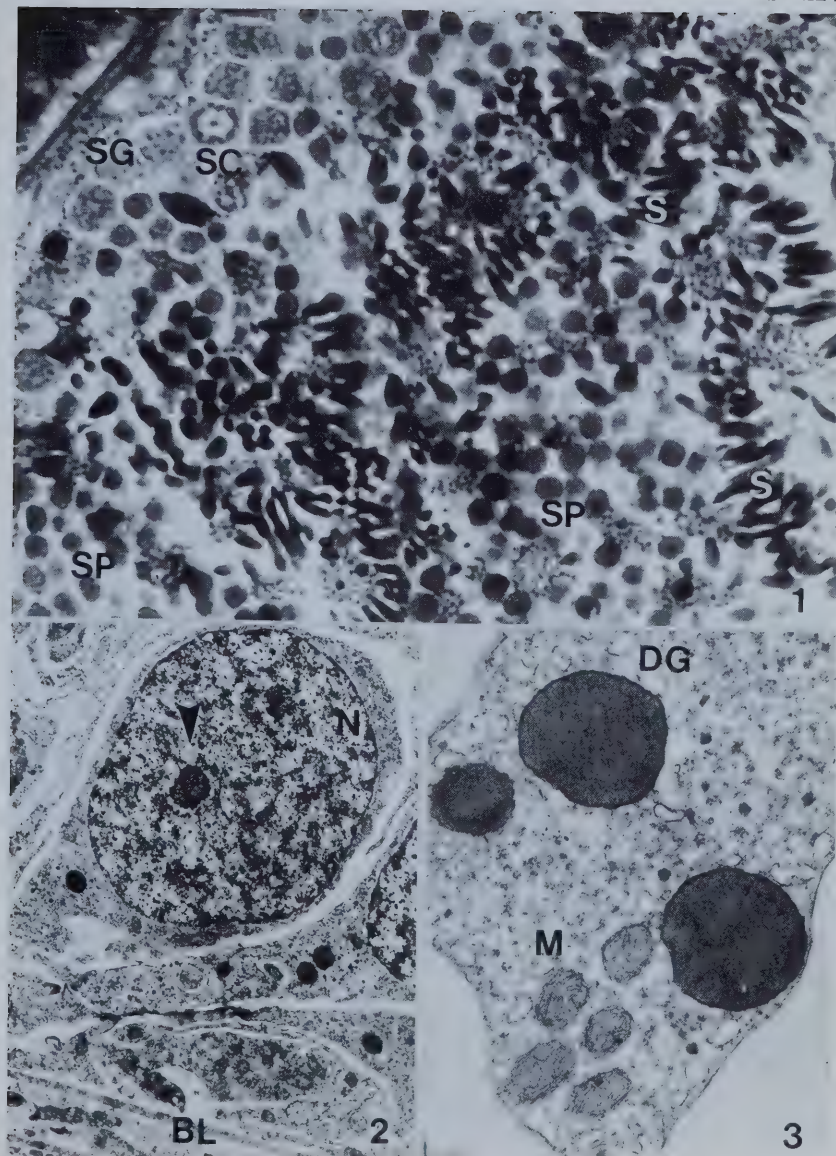
Fig. 9. Section through a spermatocyte at the flagellar region. DC, distal centriole; G, Golgi complex; PC, proximal centriole; SR, striated rootlet associated with the proximal and distal centrioles. *Cucumaria*, 34 000  $\times$ . Fig. 10. Section through the satellite of the distal centriole in *Cucumaria* spermatocyte. Radiating from the nine sets of triplet tubules of the centriole are nine primary satellite projections (three are shown). Each of these branches into two secondary branches, which in turn branch into numerous tertiary projections that form a tight network with adjacent tertiary projections. 59 000  $\times$ . Fig. 11. Membrane-bound proacrosomal granules in close association with cisternae of the Golgi complex of a spermatocyte. *Cucumaria*, 32 500  $\times$ . Fig. 12. Electron micrograph showing the assembly of a daughter centriole (above) in general region of the mother (below). Both structures lie close to cisternae of Golgi complex. *Cucumaria*, 38 500  $\times$ . Fig. 13. Section through centriolar region of *Leptosynapta* spermatocyte showing satellite projections of the distal centriole and the striated rootlet. 19 000  $\times$ . Fig. 14. Cytoplasmic region of spermatocyte containing a densely stained chromatoid body in close proximity to numerous small ovoid mitochondria and the nuclear envelope. Arrow, nuclear envelope. *Leptosynapta*, 45 000  $\times$ .

Fig. 15. Maturing spermatid of *Cucumaria*. A, acrosome; AD, acrosomal depression; M, mitochondrion; N, nucleus. 26 000  $\times$ . Fig. 16. Cross section of posterior nuclear region of *Cucumaria* spermatid. N, nucleus; black arrow, dense projection of proximal centriole; white arrow, dense chromatin layer adjacent to nuclear envelope surrounding the centriolar fossa. 50 500  $\times$ . Fig. 17. Sectional view through the centriolar fossa of *Leptosynapta* spermatid. CF, centriolar fossa; N, nucleus; P, dense fibrous projection of the proximal centriole; PC, proximal centriole. 45 000  $\times$ . Fig. 18. Longitudinal section through posterior nuclear-mitochondrial region of *Cucumaria* spermatid. M, large elongate mitochondrion; N, nucleus. 22 750  $\times$ . Fig. 19. Cytoplasmic bridge joining two *Cucumaria* spermatids. Cross section is through the posterior nuclear-mitochondrial region. N, nucleus; black arrows, dense zone adjacent to the plasma membrane of the cytoplasmic bridge. 27 000  $\times$ . Figs. 20–24. Developmental stages in the morphogenesis of the acrosomal granule in *Leptosynapta* spermatids. Refer to text for descriptions. Black arrow (small), nuclear envelope; black arrow (large), microtubules. Figs. 20–23. 43 000  $\times$ ; Fig. 24. 50 500  $\times$ . Fig. 25. Mature acrosome in anterior nuclear depression of *Leptosynapta* late spermatid. N, nucleus; PL, lamiacrosomal layer; black arrow, granule membrane; white arrow (small), centrally located concentric lamellae; white arrow (large), posterior acrosomal cup-shaped bands. 51 500  $\times$ .



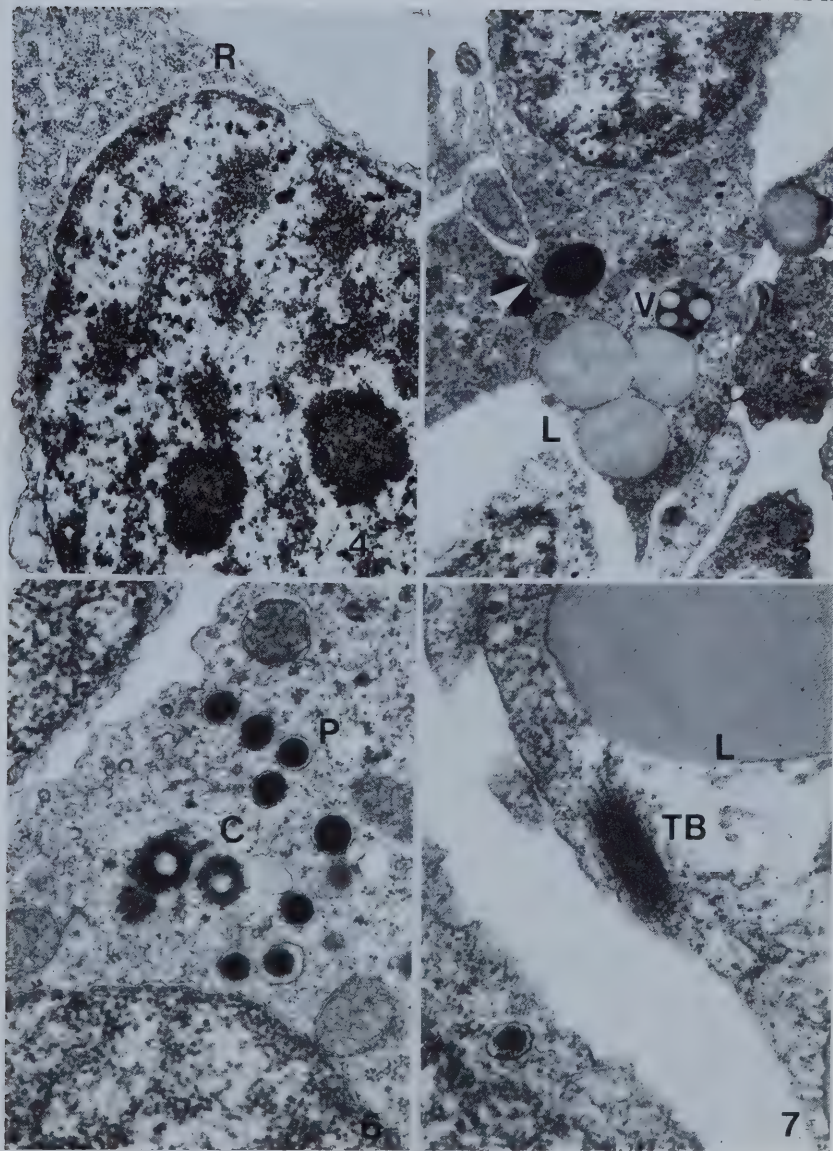


PLATE I





## PLATE II







## PLATE III

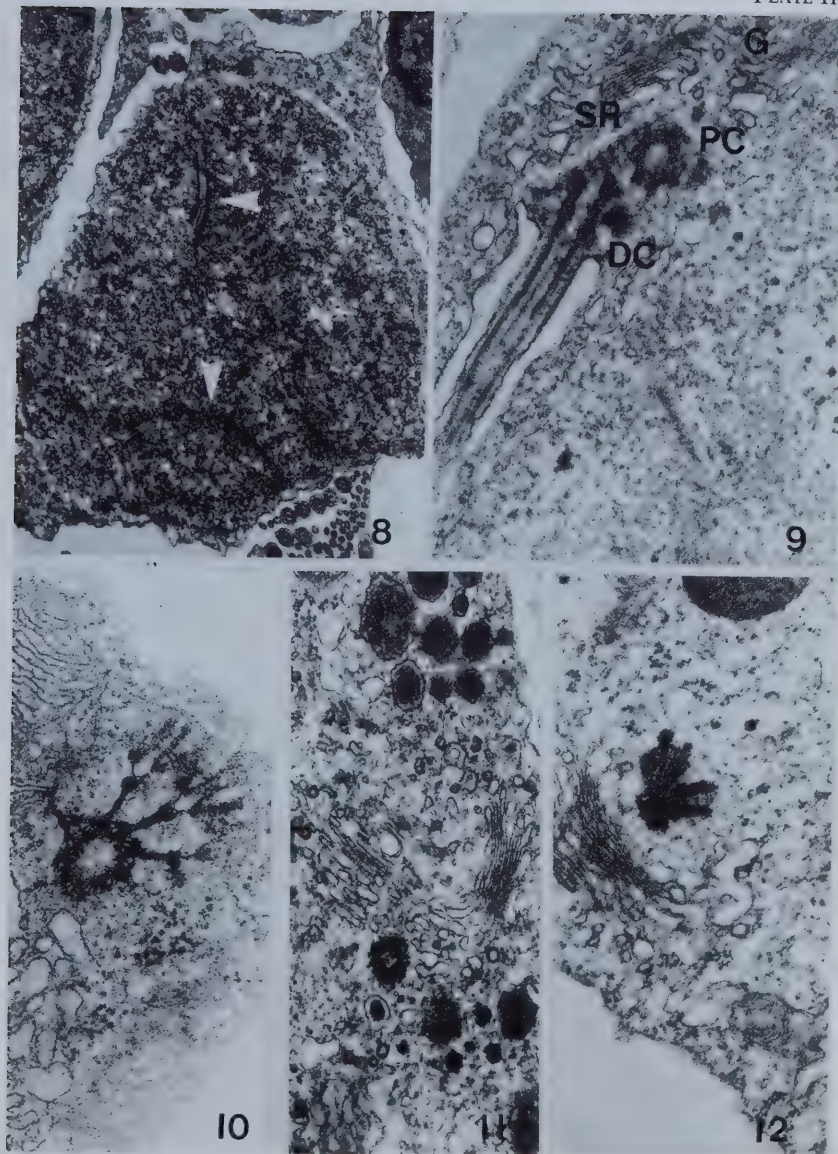




PLATE IV

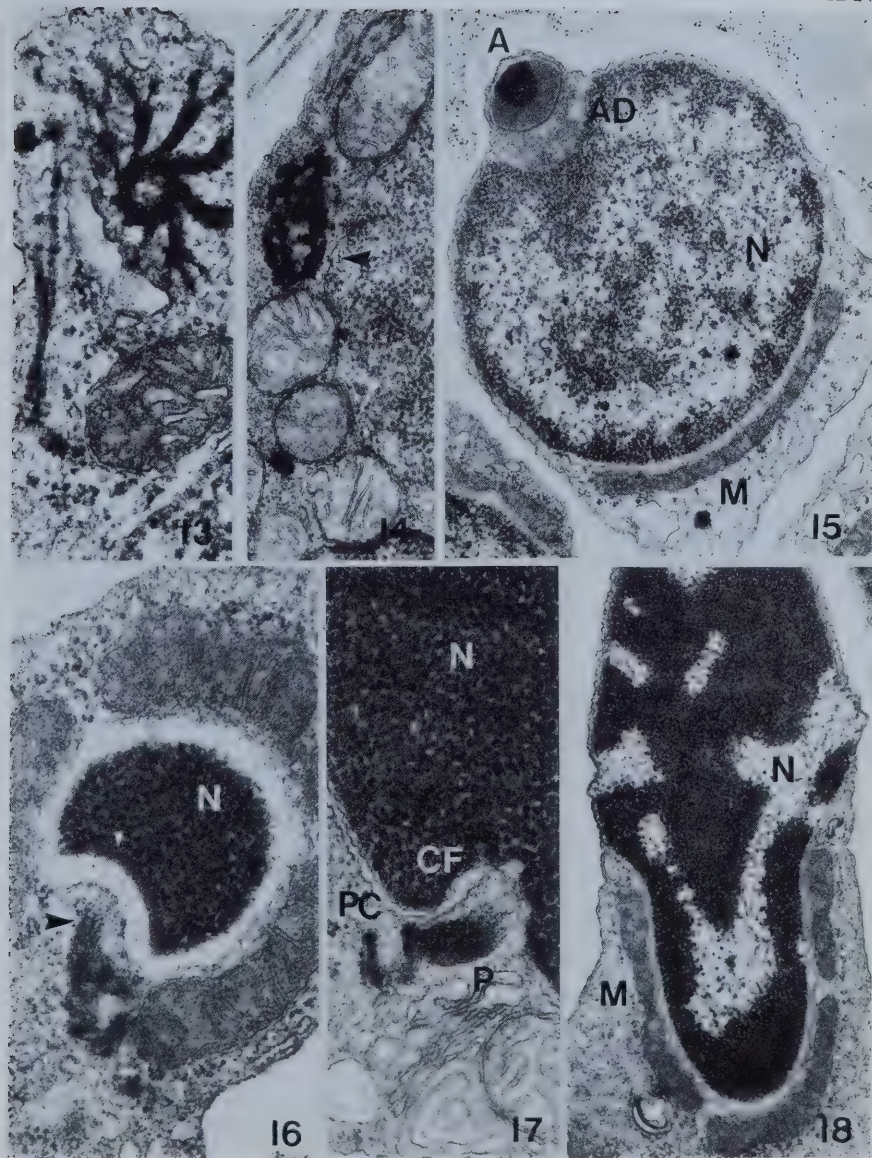
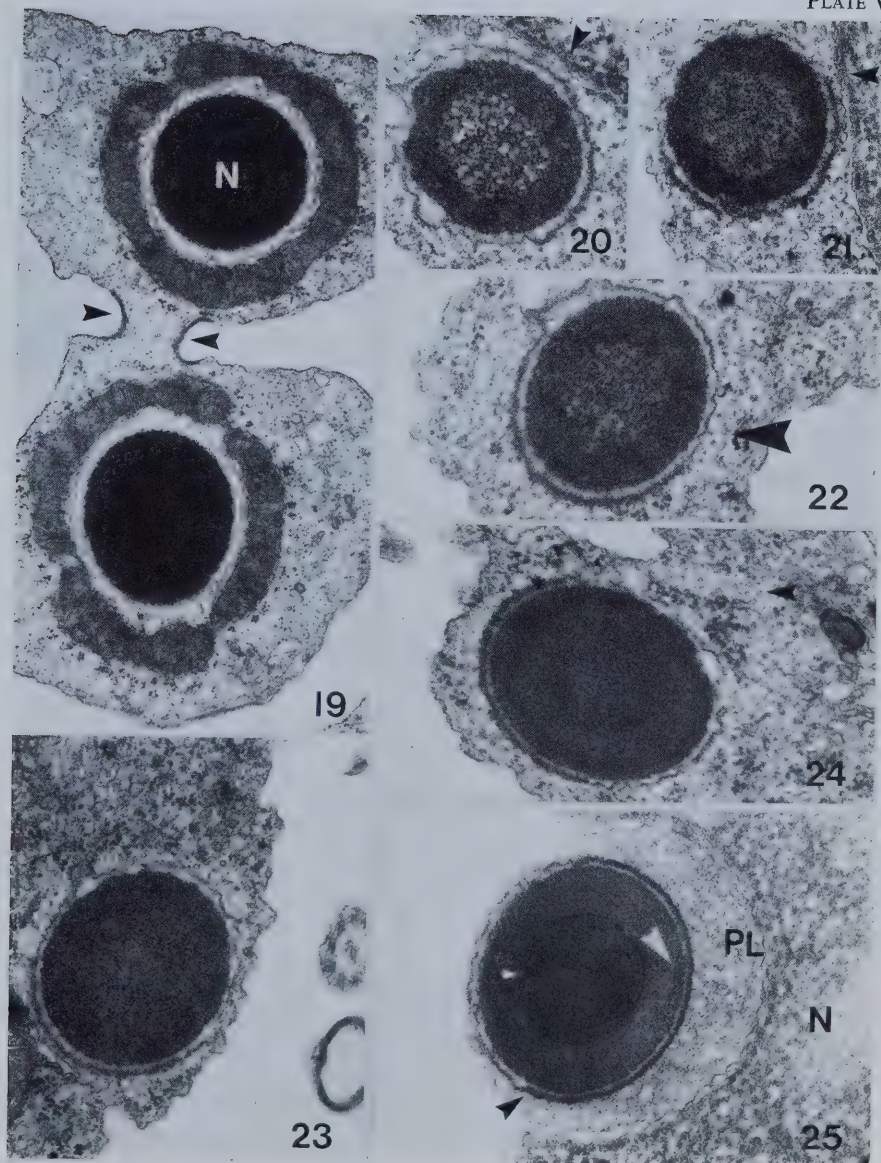






PLATE V





gradually condenses to the final state observed in late spermatids (Figs. 16, 17, 19) and forms an especially dense homogeneous layer against the inner nuclear membrane lining the centriolar fossa (Fig. 16). As spermiogenesis proceeds, nuclear indentations form at the posterior (centriolar fossa) and anterior (acrosome) regions in both species (Figs. 15, 17). The nucleus of *Leptosynapta* maintains the circular conformation throughout late spermiogenesis and remains unchanged in the mature spermatozoon (Atwood 1974). *Cucumaria* spermatids, on the other hand, undergo an elongation process which results in a torpedo-shaped nucleus which is tapered slightly at the apex and to a much greater extent at the base (Fig. 18).

Cytoplasmic bridges join spermatids in both species (Fig. 19). The relatively short bridges which are characterized by dense cytoplasmic areas adjacent to the plasma membrane contain few inclusions besides ribosomes and clear vesicles. Intercellular bridges have been observed to connect together no more than three spermatids. During the spermatid stage, excess cytoplasm that contains empty vacuoles, endoplasmic reticulum, ribosomes, and occasional microtubules is cast off. In *Cucumaria* this process normally occurs after nuclear elongation has been initiated. Cytoplasm remaining in late spermatids is confined to the mitochondrial and centriolar regions and a thin zone encompassing the lateral surfaces of the nucleus (Fig. 18).

The centrioles remain perpendicular to each other throughout spermiogenesis with the proximal centriole reaching into the centriolar fossa. Extending from the nuclear side of the proximal centriole into the fossa is a dense projection with a fibrous consistency in *Leptosynapta* (Fig. 17) and a tubular-fibrous consistency in *Cucumaria* (Fig. 16). The fibrous arms become evident as the centriolar fossa forms and become gradually less prominent as the spermatozoa mature. Longo and Anderson (1969) reporting similar projections in sea urchin spermatids have ventured that they are instrumental in the formation of the fossa. In holothurians, unlike the echinoids, the fibrous arms remain in the mature spermatozoa (Atwood 1974; Atwood and Chia 1974). The striated rootlet-like structures observed extending from the distal centriole in spermatocytes appear to degenerate by the late spermatid stage (characterized by nuclear con-

densation in both species and nuclear elongation in *Cucumaria*).

At the early spermatid stage the flagellum normally projects from the cell at about a 40° angle. After sperm maturation is completed the flagellum lies at a right angle to the mitochondrial region. The single Golgi complex observed in the mitochondrial area of both species (Fig. 11) becomes less prominent with time and is completely lacking in the mature spermatozoon of *Cucumaria*; however, the complex remains in a reduced form in *Leptosynapta*.

The small ovoid mitochondria in the basal cytoplasm of spermatogonia and spermatocytes appear as larger elongate structures in the spermatid (Figs. 15, 18, 19) and as a large single structure in the mature sperm. As in the spermatid, the mitochondrion of the mature spermatozoon of *Leptosynapta* lies at the base of the nucleus and extends only slightly toward the nuclear lateral surfaces. In late spermatids of *Cucumaria* the posterior nuclear region becomes tapered and elongates posteriorly into the mitochondrial region (Fig. 18). The nucleus of the mature sperm is further tapered, with the posterior one-third being completely encompassed by the single mitochondrial mass (Atwood and Chia 1974). No mitochondrial elements were noted being cast off in the excess cytoplasm of either species.

Formation of the acrosomal granule becomes evident in the early spermatid stage. It is presumed that the proacrosomal granules noted during earlier stages (Figs. 6, 11) coalesce to form the resulting granule. The granule begins morphogenesis in the basal cytoplasmic region and migrates to the apex of the cell by the late spermatid stage. Occasional microtubules have been noted in close association with the granule and possibly aid in migration through the cytoplasm (Figs. 22, 24). The initial granule of *Leptosynapta* is irregularly circular, surrounded by a limiting membrane, and consists of a heterogeneous material distinctly segregated into a dense reticular peripheral zone and a less dense vesicular central zone (Fig. 20). The region of the limiting membrane that will come to lie on the nuclear side of the granule (once situated in the nuclear acrosomal depression) appears to be overlaid with a thin layer of dense material (Figs. 20–25). During migration toward the apex of the cell, this side of the granule remains in





close association with the nuclear envelope (Figs. 20, 21). As the granule matures, the central vesicular structures gradually break down, forming a zone composed of a homogeneous material slightly less electron dense than that of the peripheral zone, which has become correspondingly less reticular in nature (Figs. 21, 22). At one stage during maturation the granule material becomes relatively homogeneous, with no clear distinction between the peripheral and central zones (Fig. 23). As morphogenesis continues, the granule becomes slightly larger in diameter and relatively circular in shape and develops four or five concentric dense bands (lamellae) in the central zone (Fig. 24). Initially these bands are indistinct and measure about  $0.01\ \mu$  in width. The mature granule (maturation is completed before final positioning in the nuclear acrosomal depression), measuring  $0.7\ \mu$  in diameter, contains five dense concentric lamellae, each measuring about  $0.02\ \mu$  in width, alternating with areas of much less electron-dense material. The remaining peripheral material is slightly less dense than the lamellae (Fig. 25). Two electron-dense, cup-shaped bands occur in the posterior region of the granule (Fig. 25) and are probably involved in the acrosomal reaction (Dan and Hagiwara 1967).

Encircling the acrosomal granule, within the nuclear depression, is a periacrosomal layer of homogeneous reticular material (Fig. 25). The periacrosomal material appears to originate from the cytoplasm lying in the region of the nuclear depression. The acrosomal granule was examined in various stages of migration and was never observed to be accompanied by material having the same consistency as that of the periacrosomal layer.

In *Cucumaria* the initial granule is irregularly circular and enclosed by a limiting membrane and consists of a dense homogeneous material with a reticular (fibrous) consistency. During migration toward the apical surface of the spermatid the granule material loses the reticular appearance and becomes less electron dense. The granule, by the time it reaches the nuclear region destined to become the acrosomal depression, measures  $0.6\ \mu$  in width and  $0.5\ \mu$  in length and consists of a homogeneous material except for an electron-dense sphere displaced anteriorly (Fig. 15). A number of small folds in the limiting membrane can frequently be observed at the

apex of the granule, giving a serrated effect to the anterior end of the acrosome. The posterior bands of the granule and the periacrosomal layer are basically similar to those described for *Leptosynapta*.

Except for the basal cytoplasmic area containing the centrioles, mitochondrion, and acrosomal granule, the cytoplasm of spermatids of both *Leptosynapta* and *Cucumaria* is void of microtubules. It is conceivable that the migration of the acrosomal granule is facilitated by tubules; however, because of the few numbers and the lack of definite orientation, it is doubtful. Since no microtubules are present in the cytoplasm directly adjacent to the nuclear envelope at the time of nuclear elongation in *Cucumaria*, neither does it seem likely that they play a role in initiating the elongation process. It is possible, however, that the few tubules present facilitate elongation by redistributing cytoplasm to the posterior portion of the spermatid. Nuclear elongation in *Cucumaria* is probably due to internal condensation of the chromatin. Various views on the role of microtubules in the process of nuclear elongation have recently been published (Fawcett *et al.* 1971; Ferraguti and Lanzavecchia 1971; Lanzavecchia and Donin 1972).

### Discussion

Two theories exist to explain the final shape and size of the mitochondrion observed in late spermatids. The first possibility is that the mitochondria of early stages fuse to form one single organelle, as reported by various authors (Potswald 1967; Longo and Anderson 1969). A second possibility is that only one mitochondrion exists in the cell throughout the entire spermatogenic process. The mitochondrion would, therefore, in the early germinal cell be highly branched, consisting of numerous, greatly folded, tubular units. This model would explain the many small ovoid mitochondria noted in sections of the earlier stages of spermatogenesis. It is then conceivable that as the germinal cell matures the mitochondrion condenses into a single compact organelle lying at the base of the nucleus. Preliminary data obtained from serial sections of early spermatids of *Leptosynapta* indicate that this model is feasible. Hoffmann and Avers (1973) have recently shown that a single tubular mitochondrion rather than numerous separate units



exists in the yeast cell and they have indicated that a similar situation is observed in mammalian cells. If, in fact, a single mitochondrion is the case, then what is the functional significance of the morphological transformation observed in the mitochondrial elements during spermatogenesis?

Morphogenesis of the acrosome has been studied in a wide variety of organisms (Dan and Sirakami 1971; Stanley 1971; Reed and Stanley 1972) and the process of development appears to be equally as variable. In all reported species, however, it is agreed that the initial granule is the result of fusion of proacrosomal vesicles packaged by the Golgi complex. The major variations in the process of morphogenesis appear to occur in the consistency of the materials within the granule, formation of extragranule structures, and the conformation of the acrosome. Dan (1970) has discussed much of the existing literature concerning these events.

To date, very few accounts are available that deal with echinoderm granule formation. The early granule of *Asterina pectinifera* (Asteroidea) contains a central region of sparse, randomly oriented, dense material surrounded by a region of material in a radial micellar arrangement. As spermiogenesis proceeds, the centrally located material condenses and becomes more electron dense than the peripheral region (Dan and Sirakami 1971). The initial granule in *Strongylocentrotus purpuratus* and *Arbacia punctulata* (Echinoidea) appears as a membrane-bound vesicle void of electron-dense materials. At late spermiogenesis the granule consists of a fine granular homogeneous matrix which contains no characteristic substructure (Longo and Anderson 1969). Acrosomal formation in *Cucumaria* seems to be fairly similar to that observed in asteroids, whereas *Leptosynapta* presents a case quite unique to the echinoderms. Without using histochemical techniques coupled with fertilization experimentation, it will be impossible to assign functional significance to the concentric lamellae of the *Leptosynapta* granule. The thin layer of dense material occurring on the ad-nuclear surface of the granule-limiting membrane has also been reported in asteroids (Dan and Sirakami 1971); however, there appears to be no association during migration between this region of the granule and the nuclear envelope as presently reported in holothurians.

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### Chapter III

#### SPERMATOZOOM OF *LEPTOSYNAPTA CLARKI*





## Fine Structure of the Spermatozoon of the Sea Cucumber, *Leptosynapta clarki* (Echinodermata: Holothuroidea)\*

David G. Atwood

Department of Zoology, University of Alberta, Edmonton, Alberta, Canada

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**Summary.** The spermatozoon of the holothurian *Leptosynapta clarki* has a small circular head measuring about  $3.0\ \mu$  at the greatest diameter, a midpiece containing a single mitochondrion and a tail flagellum measuring between  $35\ \mu$  and  $45\ \mu$  in length. The acrosomal region contains a granule measuring  $0.7\ \mu$  in diameter which consists of electron dense material arranged in concentric lamellae. Five concentric very electron dense lamellae alternate with areas of much less electron dense material in the central region of the granule. This granule rests in an anterior nuclear depression.

The nucleus is circular in shape and contains one or two unbound vacuoles which frequently contain a fine granular material. Posteriorly the nucleus is bounded by a large mitochondrion and an occasional Golgi complex. The proximal centriole which contains a lateral arm of dense material lies in a deep fossa projecting into the nucleus. The distal centriole lies posteriorly in the mitochondrial mass and gives rise to nine satellite projections and their Y-shaped connective extensions.

The tail contains the  $9 + 2$  tubule arrangement and tapers at its distal end.

**Key words:** Spermatozoon — Holothuroidea (*Leptosynapta clarki*) — Ultrastructure.

### Introduction

Fine structural studies on spermatozoon morphology have been published on a relatively few number of echinoderm species. The majority of these studies (usually concerned with the morphology and reactivity of the acrosomal region) have dealt with the classes Echinoidea (Afzelius, 1955, 1957; Anderson, 1968a, 1968b, 1968c; Bernstein, 1962; Dan, 1967, 1970; Dan *et al.*, 1962, 1964; Fawcett, 1970; Franklin, 1965; Inoue *et al.*, 1970; Longo and Anderson, 1968, 1969) and Asteroidea (Bernstein and Fehrenbaker, 1960; Dan, 1960, 1967, 1970; Dan and Hagiwara, 1967; Dan *et al.*, 1962; Dan and Sirakami, 1971; Hagiwara and Dan, 1969; Hagiwara *et al.*, 1967; Summers, 1972). Fragmentary studies have been reported in the classes Ophiuroidea (Dan, 1967, 1970), Crinoidea (Dan, 1967, 1970) and Holothuroidea (Dan, 1967; Summers, 1972). To date, no detailed published account is available concerning the ultrastructure of the spermatozoon of the echinoderm class Holothuroidea. Accordingly, the present study describes the fine structural morphology of the spermatozoon of the holothurian *Leptosynapta clarki*.

### Materials and Methods

Male specimens of *Leptosynapta clarki* were collected in November 1972, from the sandy substratum at the mouth of False Bay, Friday Harbor, Washington, U.S.A. Testes were immediately removed from the mature adults and fixed in a glutaraldehyde- $H_2O_2$  mixture

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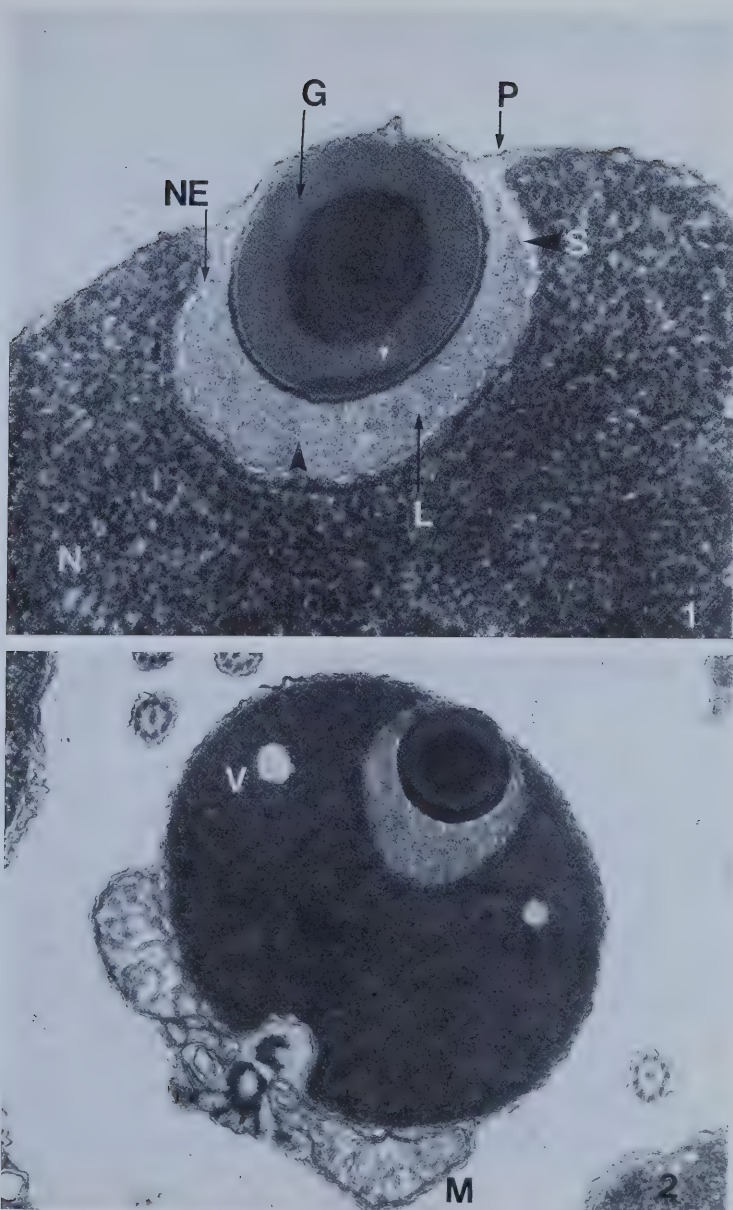


Fig. 1. Longitudinal section of acrosomal region of *Leptosynapta* spermatozoon.  $\times 73500$ . *G* acrosomal granule containing five electron dense concentric lamellae, *L* subacrosomal layer, *N* nucleus, *NE* outer membrane of nuclear envelope, *P* plasma membrane, *S* irregular spaces between layers of nuclear envelope, *black arrow* fibrous region of the subacrosomal layer, *white arrow* two posterior dense bands of acrosomal granule



### Fine Structure of *Leptosynapta* Sperm

prepared as follows. Twenty-five per cent glutaraldehyde (Fisher Scientific Company) is diluted to a 2.5% solution buffered to pH 7.6 with 0.34 M sodium chloride and 0.4 M phosphate buffer at room temperature. Thirty per cent  $H_2O_2$  (Fisher Scientific Company) is added with continuous stirring in the amount of 10 drops. Fixation was for  $2\frac{1}{2}$  hr at room temperature. Tissues were then passed into 2.5% glutaraldehyde (pH 7.6) for  $1\frac{1}{2}$  hr at room temperature, washed for 1 hr in buffer with four changes, and postfixed in 2% osmium tetroxide (in buffer) for 2 hr at room temperature.

Testicular tissues were then rinsed in 0.05 M maleic acid (pH 5.2) for 30 min with three changes and stained *en bloc* in saturated aqueous uranyl acetate for 30 min. Specimens were again rinsed in 0.05 M maleic acid, dehydrated, embedded in Araldite 502, and sectioned with a Porter-Blum-MT-2 ultramicrotome. Sections were stained with saturated aqueous uranyl acetate and 0.2% lead citrate and observed with a Philips EM 200. For light microscopy, Araldite sections were cut at  $1\ \mu$  and stained with Richardson's stain (1960).

### Results

The *Leptosynapta* spermatozoon consists of a head which is roughly spherical in shape measuring about  $3.0\ \mu$  in diameter, a large mitochondrion at the base of the nucleus and a tail ranging in length from  $35\ \mu$  to  $45\ \mu$ . For descriptive purposes the spermatozoon will be described according to the following regions: acrosomal, nuclear, mitochondrial, centriolar and tail.

#### Acrosomal Region

This region contains a large acrosomal granule measuring  $0.7\ \mu$  in diameter set in a nuclear depression measuring  $1.0\ \mu$  in width and  $0.9\ \mu$  in depth (Figs. 1, 9). The circular granule is almost completely enclosed by lateral extensions of the nucleus and the entire acrosomal region is confined by the plasma membrane which encompasses the spermatozoon. The acrosomal granule, surrounded by a bounding membrane, consists of electron dense material which in the central region is arranged in concentric lamellae (Fig. 1). Four or five such very electron dense areas (each measuring about  $0.02\ \mu$  in width) alternate with areas of much less electron dense material. The remaining area of the granule is nearly as electron dense as the concentric lamellae. Two very electron dense bands (approximately half the width of the centrally located lamellae) occasionally occur in the posterior region of the granule (Figs. 1, 9). In numerous sections these bands appear as ill-defined, incomplete membranes. Areas of much less electron dense material occur between and below these posterior bands. The bands are somewhat cup-shaped and appear to extend around the posterior region and slightly into the adjacent sides of the acrosomal granule (Fig. 1).

Completely encircling the granule is a layer of homogeneous reticular material much less electron dense than the granule (Fig. 1). This subacrosomal layer varies in thickness from  $0.3\ \mu$  around the posterior surfaces of the granule to about  $0.01\ \mu$  around the anterior surfaces and does not appear to be bound by membranes. The outer surface of this layer comes in close contact to the nuclear envelope at the posterior region and the plasma membrane at the anterior (Fig. 1). Posterior

Fig. 2. Longitudinal section of *Leptosynapta* sperm showing anterior nuclear depression, posterior centriolar fossa and two centrioles.  $\times 30000$ . *M* mitochondrion, *V* nuclear vacuoles





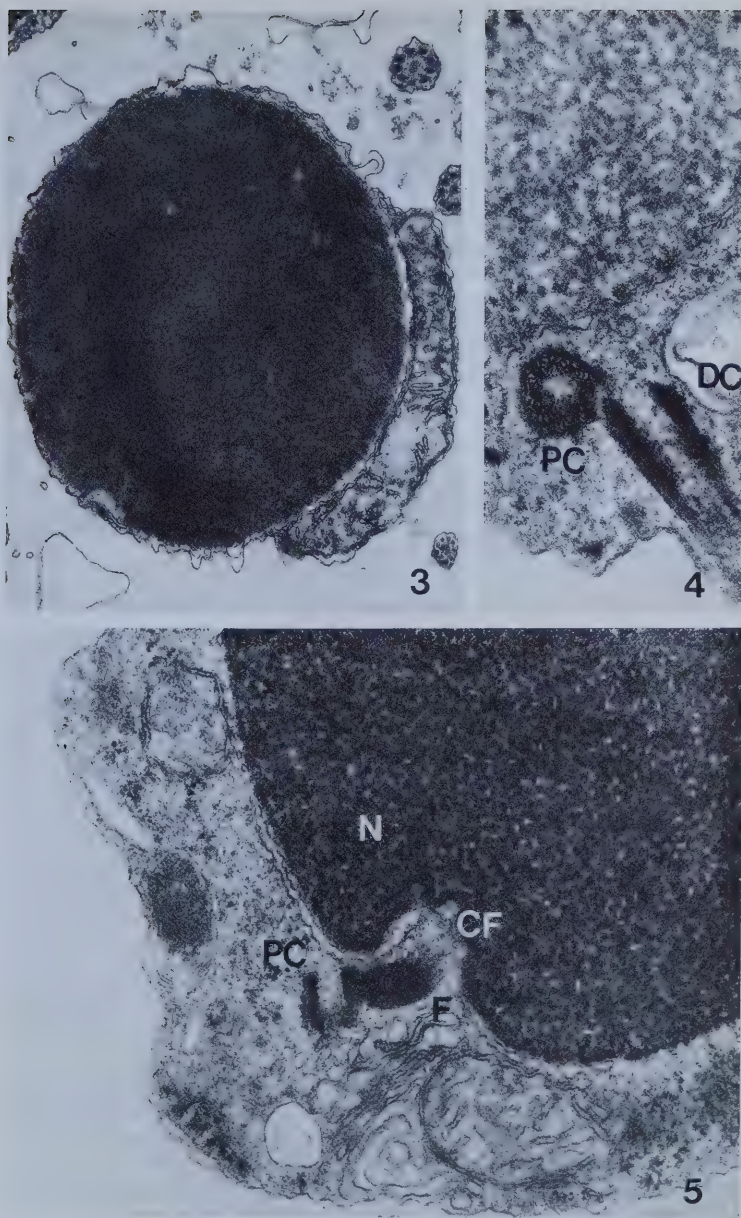


Fig. 3. Section through nucleus and mitochondrion.  $\times 27000$

Fig. 4. Longitudinal section through centriolar region of *Leptosynapta* sperm.  $\times 59000$ .

DC distal centriole, PC proximal centriole

Fig. 5. Micrograph of the posterior nuclear region.  $\times 45000$ . CF centriolar fossa, F fibrous arm of proximal centriole, N nucleus, PC proximal centriole





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to the acrosomal granule (beneath the two posterior electron dense bands) the subacrosomal layer becomes less electron dense and appears to contain a small quantity of fibrous material (Fig. 1). This ill-defined area forms no obvious subdepression and is infrequently observed. Small irregular spaces occur around the outer surfaces of the subacrosomal layer and appear to be between the membranes of the nuclear envelope (Fig. 1). These spaces have been shown with several different fixatives and are not believed to be artifacts.

### Nuclear Region

The nucleus of *Leptosynapta* spermatozoa is roughly circular in shape measuring about  $2.9\ \mu$  in width (Figs. 2, 3). The anterior-posterior axis is somewhat shorter due to the acrosomal depression and measures about  $1.4\ \mu$ . One or two vacuoles, unbound by membranes, are present within the nucleoplasm and occasionally contain a fine granular material (Fig. 2). The anterior surface of the nucleus is indented by the acrosomal region and the posterior surface by the centriolar fossa which contains a proximal centriole (Fig. 2).

### Mitochondrial Region

This region contains a large single mitochondrion which rather than forming a depression wraps around the posterior surface of the nucleus (Figs. 3, 9). A distinct Golgi complex is occasionally encountered in close proximity to the distal centriole. Electron-dense granules possibly representing glycogen infrequently occur around the posterior surfaces of the mitochondrion. The above structures are embedded in a narrow cytoplasmic matrix (Figs. 2, 3).

### Centriolar Region

Two centrioles (proximal and distal), each containing nine sets of three tubules, are located posterior to the nucleus within the mitochondrial region (Fig. 4). The proximal centriole is oriented perpendicular to the longitudinal axis of the spermatozoon and lies within a fossa which extends into the base of the nucleus (Fig. 2). There appears to be a dense fibrous projection radiating from the nuclear side of the proximal centriole and extending deep into the fossa. This projection appears as a lateral arm when viewing a longitudinal section of the centriole (Fig. 5).

The distal centriole (basal body) which is connected to a series of satellite projections and Y-shaped membrane doublet connectives is separated by a distance of  $0.08\ \mu$  from the proximal centriole. A cross section through the anterior region of the distal centriole reveals the nine sets of tubules which extend into the tail as the peripheral tubule doublets. Centriolar satellite projections and connectives are not evident at this level (Fig. 9). Just distal to this region is the satellite which consists of nine radiating fibers each of which branches into two secondary fibers and in turn branches into fine tertiary fibers which form a network with adjacent tertiary fibers (Figs. 6, 7). The network, with which microtubules are closely associated, is very extensive and lies in close proximity to the mitochondrial membranes (Fig. 6). Distal to this level the centriolar satellite continues into the tail in the form of Y-shaped membrane connectives.



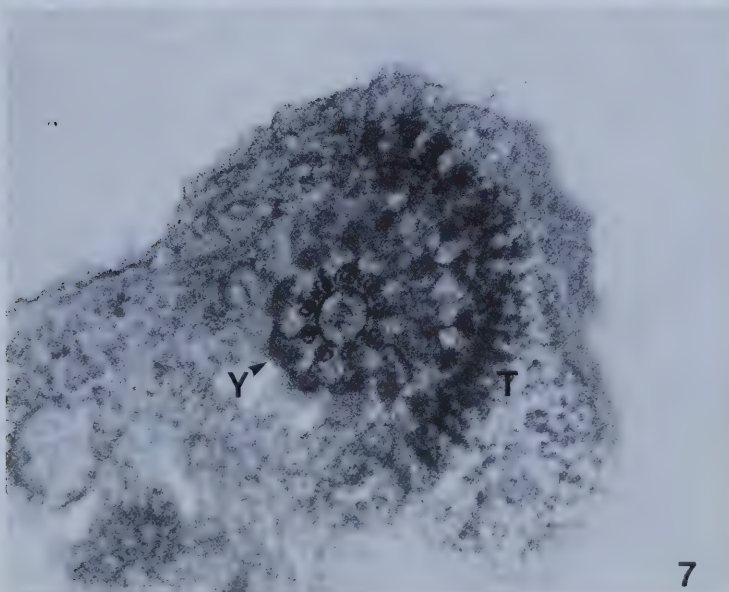
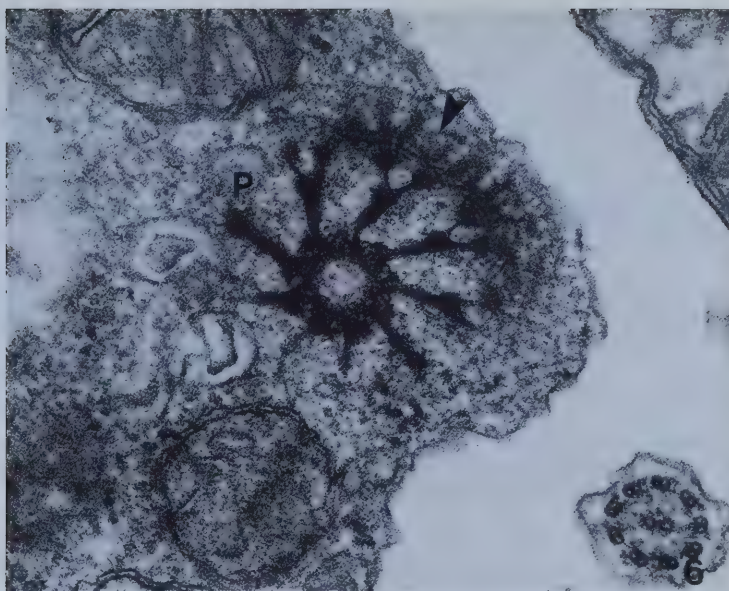


Fig. 6. Cross section through distal centriole containing the nine primary satellite projections, each of which branches into two secondary fibers.  $\times 81000$ . *P* satellite projections, *arrow* tertiary fiber network and associated microtubules. Note the close association between the tertiary network and mitochondrial membranes

Fig. 7. Oblique section through distal centriolar satellite complex.  $\times 81000$ . *T* tertiary satellite fibers, *Y* Y-shaped membrane connectives





Fig. 8. Cross sections of the *Leptosynapta* sperm tail.  $\times 132500$ . *A* arms of the A-tubules, *S* spokes radiating from the central region and extending to the A-tubules, arrow distal region of tail containing nine single peripheral tubules surrounding two single central tubules.

#### *Tail Region*

*Leptosynapta* sperm tails are  $35\ \mu$  to  $45\ \mu$  in length and about  $0.2\ \mu$  to  $0.3\ \mu$  in diameter. The most proximal region of the tail contains the typical nine peripheral doublet tubules which surround the two single central tubules. The A-tubule of each doublet is concentric whereas the B-tubule is more crescent-shaped and somewhat smaller in size. At this level no arms or spokes are observed; however, Y-shaped membrane doublet connectives are evident connecting each A-tubule to the scalloped plasmalemma (Fig. 7). A thin filament connects all nine A-tubules forming a nine-sided configuration. The Y-shaped connectives appear to be distal extensions of the satellite projections as seen in an oblique section through the base of the flagellum (Fig. 7).

Just distal to the above region the A-tubule of each peripheral doublet develops small arms (Fig. 8). The radiating arms of the A-tubule of the number five doublet occasionally come into close proximity with doublet number six; however, no actual contact was observed (Afzelius, 1959, for number system). There is an equal distance maintained between the remaining seven peripheral doublets. Nine ill-defined spokes radiate from the region of the two central tubules and terminate in close proximity to the A-tubules of the peripheral doublets (Fig. 8).

At the distal region of the tail the number of tubules is reduced to 11, nine peripheral and two central (Figs. 8, 9). No arms or spokes are evident in this region. The tail tapers at the most distal tip and only the two central tubules remain.





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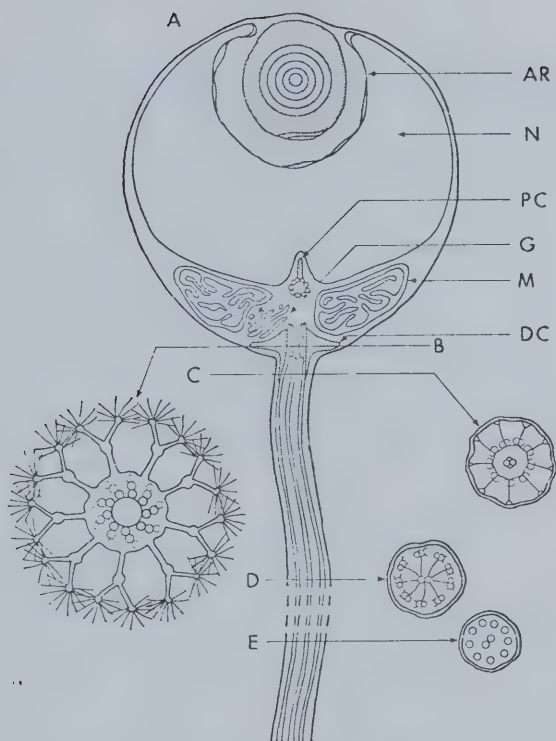


Fig. 9. Diagram of the *Leptosynapta* spermatozoon. *A* longitudinal diagram of intact sperm, *B* cross-sectional diagram through the distal centriole and satellite projections, *C* section through proximal region of tail containing Y-shaped membrane connectives, *D* section through mid-region of tail containing arms and spokes, *E* section through distal region of tail, *AR* acrosomal region, *DC* distal centriole, *G* Golgi complex, *M* mitochondrion, *N* nucleus, *PC* proximal centriole

### Discussion

The acrosomal region of the holothurian (*Leptosynapta*) spermatozoa is similar to that described for asteroids (Dan, 1970; Dan and Hagiwara, 1967; Hagiwara, *et al.*, 1967), echinoids (Bernstein, 1962; Dan *et al.*, 1964; Franklin, 1965), and erinoids (Dan, 1970; Holland, unpublished) with several variations. No comparative ophiuroid literature is currently available.

The spherical asteroid sperm head contains an acrosomal granule, not bound by a continuous membrane, which is asymmetrical with the anterior surface (containing a small crater) being wider than the posterior (Hagiwara *et al.*, 1967). Bernstein (1962) and Longo and Anderson (1969) have suggested that the spherical granule of echinoids is surrounded by a delimited membrane. Dan *et al.* (1964) maintain that instead of a unit membrane surrounding the echinoid granule there



### Fine Structure of *Leptosynapta* Sperm

is a composite of several separate components, which interact to produce the membranous structure bounding the acrosomal process during the acrosomal reaction. Holland (unpublished) has suggested that the spherical granule of crinoids is also bounded by a membrane as is reported in holothurians (Fig. 1).

The asteroid granule contains, instead of concentric dense bands as in *Leptosynapta* (Fig. 1), a central electron dense region surrounded by a mass of material arranged in sparse radially oriented strands (Hagiwara *et al.*, 1967). The granule of echinoids is composed of a homogenous particulate material not arranged in an organized manner (Dan *et al.*, 1964). Crinoid acrosomal granules appear to vary in composition in different species. *Florometra* has a very dense granular component filling the anterior fifth of the granule and a less dense granular component occupying the posterior four-fifths (Holland, unpublished).

At the posterior region of the asteroid acrosomal granule a dense area occurs which consists of an inner component bounded by two dense layers and an outer component bounded by three. Dan and Hagiwara (1967) have called these layers the "primary membrane precursors" which are involved in the initial stages of the acrosomal reaction. Evidently, the dense posterior bands observed in the present study correspond to this laminate plate. Homologous regions have also been identified in echinoids (Dan *et al.*, 1964) and crinoids (Dan, 1970).

Beneath the asteroid granule a subdepression, containing a loose mass of medium electron-dense material which will give rise to the axial fiber of the acrosomal process, extends into the nucleus (Dan and Hagiwara, 1967). This area is represented in the holothurian by an inconspicuous region not forming a nuclear subdepression (Fig. 1). Echinoids (Dan *et al.*, 1964) and crinoids (Dan, 1970) have distinct fibrous subdepressions as described in asteroids. On either side of this subdepression, some echinoids contain elongate peg-like structures extending from the distal edge of the nuclear membrane into the space between the plasma membrane and the flank of the acrosomal granule (Dan *et al.*, 1964). No similar structures have been reported in the other echinoderm classes.

All asteroid (Bernstein and Fehrenbaker, 1960), echinoid (Longo and Anderson, 1969), crinoid (Holland, unpublished), ophiuroid (Dan, 1970) and holothurian (present paper) species examined have sperm containing a single large mitochondrion at the base of the nucleus. In all cases the mitochondrion wraps around the nuclear base instead of forming a depression in the nucleus.

The dense fibrous arm extending into the centriolar fossa from the proximal centriole (Fig. 5) of *Leptosynapta* has been reported in several other species (Daniels *et al.*, 1971; Longo and Anderson, 1969; Potswald, 1967), Longo and Anderson (1969) in sea urchins and Potswald (1967) in *Spirorbis* (Polychaeta) observed such a fibrous complex projecting from the proximal centriole only during the spermatid stage of spermiogenesis. Daniels *et al.* (1971) reported a similar radiating arm in the mature sperm of *Crassostrea* (oyster) oriented parallel to the proximal centriole. Longo and Anderson (1969) observed that the formation of the centriolar fossa (sea urchins) was always in association with the fibrous complex and that after formation of the fossa the fibrous arm degenerated.

Centriolar (distal) satellites in echinoderm spermatozoa have been reported in the classes Echinoidea (Longo and Anderson, 1968, 1969), Asteroidea (Summers, 1972) and Holothuroidea (Summers, 1972). Examination of micrographs of the



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distal centriolar region of the asteroid, *Ctenodiscus crispatus* (Summers, 1972), reveals that each satellite projection branches into two fibers as in *Leptosynapta*. No comparable work is available in other echinoderm species. However, throughout the animal phyla morphological variation does occur. The satellites of *Pennaria* (Cnidaria) are club-shaped at their distal ends (Summers, 1970) and in the jellyfish *Nausithoe* each primary fiber gives rise to a straight middle secondary fiber and two lateral secondary fibers which form a network with neighboring secondary fibers (Afzelius and Franzen, 1971).

The results from the present study agree with the centriolar satellite complex model set forth by Summers (1972) for *Pennaria tiarella*. It is obvious that also in *Leptosynapta* the Y-shaped membrane connectives of the anterior tail region are basal extensions of the nine satellite projections of the distal centriole (Fig. 7). It has been suggested that the satellite complex functions in providing structural support for the distal centriolar-flagellar region (Szollosi, 1964) and possibly in the transporting of ATP from the mitochondria to the flagellum (Summers, 1972).

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David G. Atwood  
 Department of Zoology  
 University of Alberta  
 Edmonton, Alberta T6G 2E1  
 Canada





## Chapter .IV

### SPERMATOZOON OF *CUCUMARIA LUBRICA*



## Fine structure of an unusual spermatozoon of a brooding sea cucumber, *Cucumaria lubrica*

DAVID G. ATWOOD AND FU-SHIANG CHIA

Department of Zoology, University of Alberta, Edmonton, Alberta

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The sperm of *Cucumaria lubrica* consists of an elongated head, 1.5 microns ( $\mu$ ) in diameter and 5.2  $\mu$  in length; a mitochondrial midpiece of 1.9  $\mu$  in length; and a tail of 65  $\mu$ . The acrosome contains an irregularly circular granule which is located in an anterior nuclear depression. The granule is surrounded by a complete limiting membrane in most of the sperm examined. Occasional sperm contain granules surrounded by incomplete membranes. The acrosomal granule consists of a homogeneous material except for an electron-dense sphere displaced anteriorly. A subacrosomal layer consisting of a homogeneous reticular material encircles the granule. This layer contains several vesicular structures and a fibrous component immediately posterior to the granule.

The nucleus measures 6.8  $\mu$  in length and 1.4  $\mu$  at its greatest diameter, but tapers to a diameter of 0.5  $\mu$  at the posterior end. A single mitochondrial mass surrounds the posterior 1.6  $\mu$  of the nucleus. The proximal and distal centrioles, lying perpendicular to each other, are found posterior to the nucleus. Microtubules are observed in close association with the satellite of the distal centriole.

The adaptive significance of this unusual sperm is discussed.

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Le spermatozoïde de *Cucumaria lubrica* est constitué d'une tête allongée de 1.5 microns ( $\mu$ ) de diamètre et 5.2  $\mu$  de longueur, d'une pièce intermédiaire mitochondriale de 1.9  $\mu$  de longueur et d'une queue de 65  $\mu$  de longueur. L'acrosome contient un granule circulaire irrégulier, localisé dans une dépression antérieure du noyau. Le granule s'entoure d'une membrane entière dans presque tous les spermatozoïdes examinés. Dans quelques cas, cependant, les granules sont entourés de membranes incomplètes. Le granule de l'acrosome se compose d'une substance homogène; on observe toutefois, vers l'avant du granule, une sphère opaque au microscope électronique. Une couche subacrosomique constituée de substance réticulée homogène entoure le granule. Cette couche contient plusieurs structures vésiculaires ainsi qu'une structure fibreuse située juste derrière le granule.

Le noyau mesure 6.8  $\mu$  de longueur et son diamètre passe de 1.4  $\mu$  à 0.5  $\mu$  vers l'extrémité postérieure. Une masse mitochondriale unique entoure les 1.6  $\mu$  postérieurs du noyau. Les centrioles proximal et distal, perpendiculaires l'un à l'autre, sont postérieurs au noyau. On observe des microtubules en relation étroite avec le satellite du centriole distal.

On discute de l'importance adaptative de ce spermatozoïde singulier. [Traduit par le journal]

### Introduction

Available information on holothurian sperm morphology, including *Stichopus regalis* and *Cucumaria planci* (Field 1893, 1895); *Mesothuria intestinalis* (Retzius 1905); *Holothuria atra* (A. L. Colwin and L. H. Colwin 1955); *Thyone briareus* (L. H. Colwin and A. L. Colwin 1955, 1956); *Parastichopus californicus* (Dan 1967); *Cucumaria elongata* (Chia and Buchanan 1969); *Cucumaria miniata* (Fontaine, personal communication); and *Leptosynapta clarki* (Everingham 1961), has shown, without exception, that the sperm head is spherical. In general, the head is positioned anteriorly to a single mitochondrial mass, while the acrosome is located in a cup-shaped depression at the apex of the nucleus.

This type of sperm has been considered as being primitive (Tuzet 1950; Franzen 1956; Fawcett 1970) and was thought to be related to the mode of external fertilization.

In an examination of the spermatozoa of *Cucumaria lubrica*, we are surprised to find that the sperm head is elongated and slightly tapered anteriorly; this proves to be the first case among holothurians to have such a sperm. In fact, among echinoderms as a whole, no other species has ever been reported, as far as we are aware, to produce an elongate, torpedo-shaped sperm. Echinoids produce a similar elongate spermatozoon which, however, differs in that the anterior end is extremely tapered.

In this report, we describe the fine structure



of this sperm and discuss the possible relationship between this morphological variation and the specific mode of reproduction in this species.

### Materials and Methods

All specimens were collected subtidally at Eagle Point, San Juan Island, Washington, in December, 1972. Testes were removed from the mature males and fixed in 2.5% glutaraldehyde (Fisher Scientific Company) with seawater (pH 8.0) for 1½ h at room temperature. The materials were then washed in two 15-min changes of 0.4 M Millonig's phosphate buffer (Millonig 1962) (pH 7.6) in 0.34 M sodium chloride, before postfixation in osmium tetroxide in 0.4 M Millonig's phosphate buffer (Millonig 1962) (pH 7.6) for 45 min at room temperature. They were embedded in Araldite 502 and sectioned with a Porter-Blum MT-2 ultramicrotome. Sections were stained with saturated aqueous uranyl acetate for 2½ h, and with 0.2% lead citrate for 3 min, then observed with a Philips EM 200. For light microscopy, Araldite sections were cut at 1 µ and stained with Richardson's stain (Richardson *et al.* 1960).

For scanning electron microscopy, sperm were pipetted directly from the dissected testes into filtered seawater and washed three times by hand centrifugation. They were then transferred to 2.5% glutaraldehyde (Fisher Scientific Company) with seawater (pH 8.0). After two 10-min washes in distilled water, the spermatozoa were transferred to 30% ethanol, dehydrated in ascending concentrations of ethanol at 10-min intervals, and finally air-dried. The dried spermatozoa were rotary-shadowed with carbon, then gold, to 75–125 Å. A Cambridge S-4 Stereo Scan scanning electron microscope was used for the examination of specimens.

### Results

The *Cucumaria* spermatozoon consists of an elongated head measuring 1.5 µ in diameter and 5.2 µ in length, a large mitochondrial mass at the base of the nucleus measuring 1.9 µ in length, and a tail ranging from 60 µ to 70 µ (Fig. 3). The spermatozoon will be described according to the following regions: acrosome, nucleus, mitochondrion, centriole, and flagellum.

#### Acrosome

This region, containing an irregularly circular acrosomal granule measuring about 0.6 µ in width and 0.5 µ in length, rests in a nuclear depression (Fig. 4). The granule, completely surrounded by a limiting membrane in most sperm, consists of a homogeneous material except for an electron-dense sphere displaced anteriorly (Fig. 4). The homogeneous acrosomal material completely fills up the area enclosed by the acrosomal membrane in most sections (Figs. 1, 2). In several sections (Fig. 4), however, the material is lacking at the apical surface of the granule. This condition is possibly due to compression of material centrally. A number of small folds of the limiting membrane can frequently be observed in the apex of the granule, giving a serrated effect to the anterior end of the acrosome. In some sperm, the limiting membrane

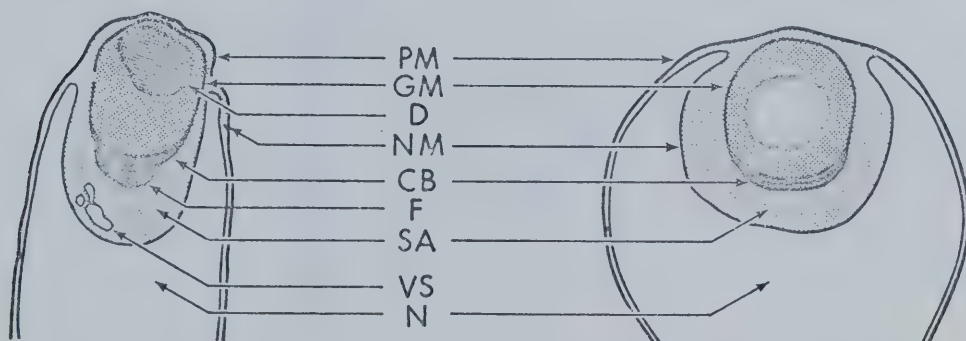


FIG. 1. Diagram showing the acrosomal region of *Cucumaria* (left) and *Leptosynapta* (right). CB, cup-shaped bands within the granule; D, electron-dense disc; F, fibrous component of subacrosomal layer; GM, acrosomal granule membrane; N, nucleus; NM, nuclear membrane; PM, plasma membrane; SA, subacrosomal layer; VS, vesicular structures.

FIG. 2. Diagram of *Cucumaria* spermatozoon. A, longitudinal section of intact sperm; B–E, cross or oblique sections through different levels as indicated; AD, anterior nuclear depression; AR, acrosomal region; CF, centriolar fossa; DA, dense arm; DC, distal centriole; G, glycogen-like particles; M, mitochondrion; N, nucleus; P, projecting arm; PC, proximal centriole.





## ATWOOD AND CHIA: SEA CUCUMBER SPERM

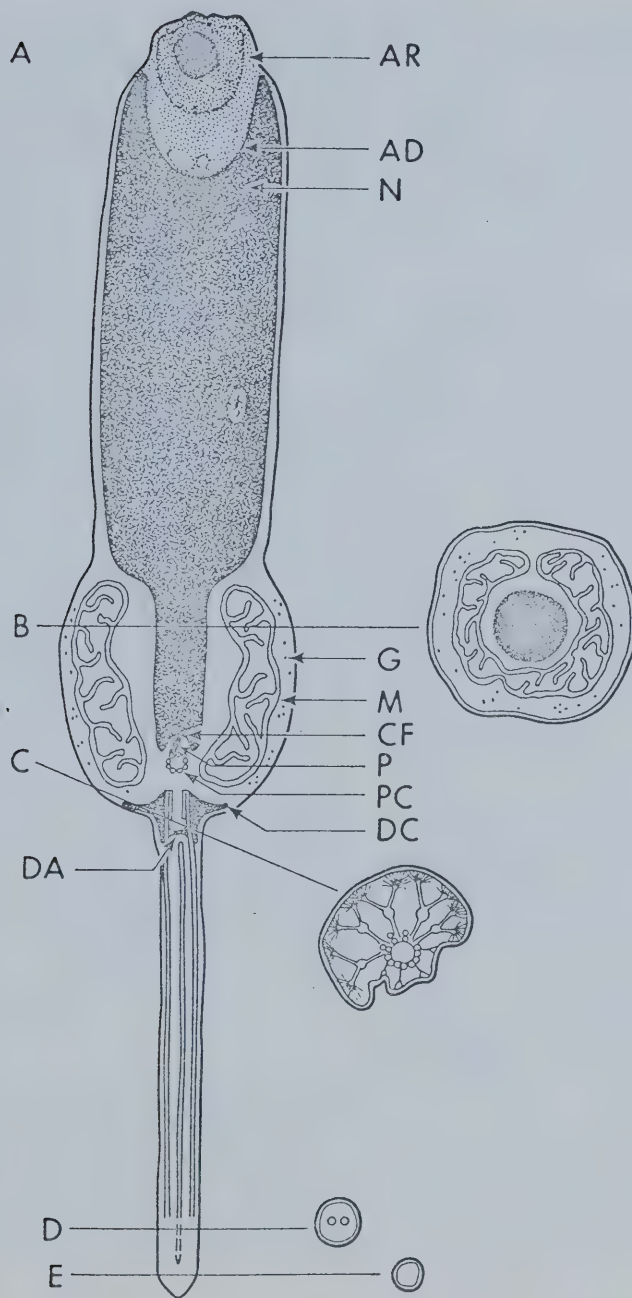


Fig. 2



along the lateral surfaces of the acrosomal granule appears to be incomplete (Figs. 1, 4). Because of the repetitive occurrence and location of these disruptions, this condition is not believed to be an artifact. Unless one of these conditions represents an immature sperm, both conditions must be considered to occur normally in this species. Comparatively speaking, the incomplete membrane situation has been observed by Dan (1967) for *Parastichopus californicus*, but complete membranes have been reported by Summers *et al.* (1971) in *Thyone* and by Atwood (unpublished observations<sup>1</sup>) in *Leptosynapta clarki* (Fig. 1).

Two electron-dense, cup-shaped bands measuring about 100 Å in width occasionally occur in the posterior region of the granule (Fig. 4). These bands appear as ill-defined, incomplete layers and extend into the adjacent sides of the granule (Figs. 1, 4). These structures have been referred to as primary membrane precursors, which have been shown to be involved in the acrosomal reaction in starfish sperm (Dan and Hagiwara 1967).

Completely encircling the granule is a homogeneous subacrosomal (periacrosomal) layer of reticular material less electron dense than the granule. This subacrosomal layer varies in thickness from 0.4 μ around the posterior end of the granule to about 80 Å around the anterior, and it is not bound by a limiting membrane (Fig. 4). Vesicular structures, numbering from one to six, are frequently observed within the posterior regions of the subacrosomal layer, lying in close proximity to the nuclear envelope (Fig. 4). Immediately posterior to the acrosomal granule, the subacrosomal layer becomes less electron dense and contains a distinct fibrous component (Figs. 1, 4).

### Nucleus

The elongated nucleus of the *Cucumaria* spermatozoon possesses a depression anteriorly to house the acrosome and a slight centriolar fossa posteriorly (Fig. 2). The nucleus measures about 6.8 μ in length and 1.4 μ at its greatest diameter. The posterior 1.6 μ of the nucleus, tapered to a diameter of 0.5 μ, is surrounded by a large mitochondrial mass (Figs. 2, 5). A small

vacuole with a fine granular material is occasionally present in the nucleus (Fig. 2).

### Mitochondrion

The single mitochondrial mass measuring about 1.9 μ in length surrounds the posterior quarter of the nucleus (Figs. 2, 5, 6). Electron-dense granules, glycogen-like particles, occur posteriorly to the mitochondrion within the cytoplasm (Fig. 6).

### Centriolar Region

The proximal and distal centrioles are positioned posteriorly to the nucleus within the mitochondrial mass (Figs. 2, 7). The proximal centriole is oriented perpendicular to the longitudinal axis of the sperm and sends out a dense projecting arm into a centriolar fossa at the base of the nucleus (Figs. 2, 8, 9). A similar situation has been observed in spermatozoa of *Leptosynapta clarki* (Atwood, unpublished observations<sup>1</sup>).

The anterior region of the distal centriole (basal body) contains the nine sets of tubules which continue into the flagellum as the peripheral tubule doublets. Distal to this area is a centriolar satellite, which consists of nine radiating fibers each of which branches into two secondary fibers and in turn branch into fine tertiary fibers (Figs. 2, 10). These tertiary fibers together form an extensive network (Fig. 10), which lies in close association with the posterior surface of the mitochondrial mass. Longitudinal views of the satellite are shown in Figs. 7, 8, and 11. Microtubules are occasionally observed in the region of the tertiary fiber network; however, they have not been conclusively shown to be an integral component of the satellite (Figs. 7, 10).

Anterior to the central doublet of the flagellum, within the satellite region, an arm of electron-dense material is found between the peripheral doublets of the flagellum (Figs. 2, 11).

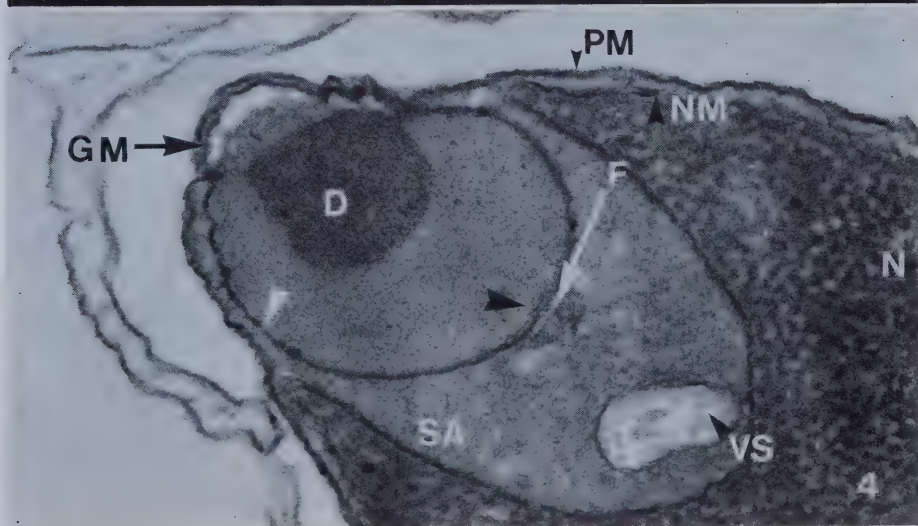
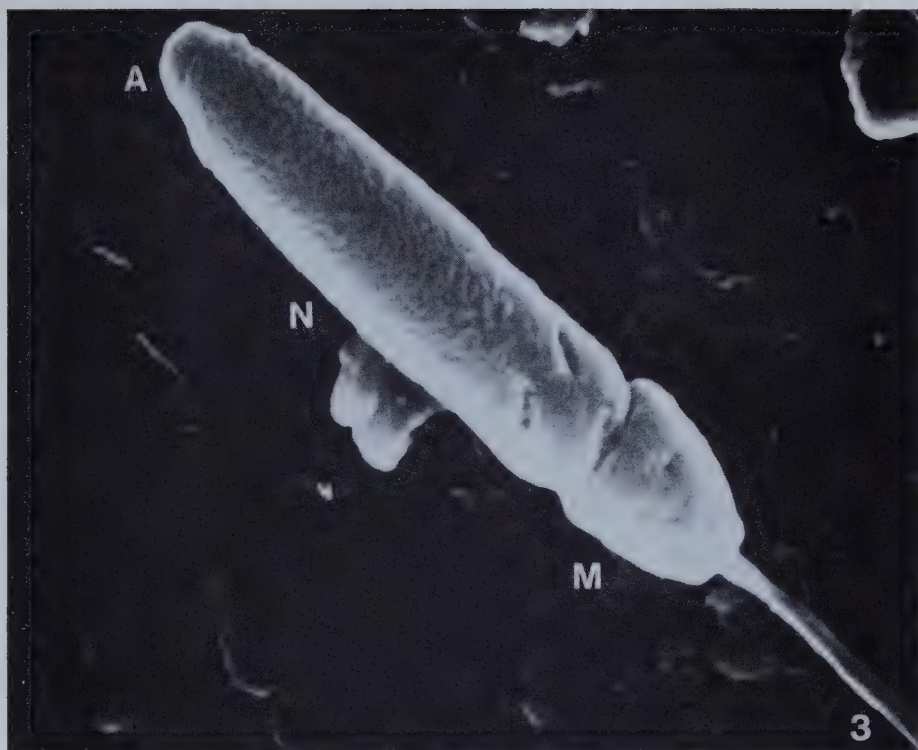
### Flagellum

Spermatozoa flagella of *Cucumaria* are 60 μ to 70 μ in length and about 0.2 μ to 0.3 μ in diameter. The anterior portion of the flagellum contains nine Y-shaped connectives, which connect the A tubules to the plasmalemma (Fig. 12). The Y-shaped connectives are distal extensions of the satellite projections of the distal centriole (Figs. 2, 12). The flagellum tapers toward the

<sup>1</sup>D. G. Atwood, Fine structure of the spermatozoon of the sea cucumber *Leptosynapta clarki* (Echinodermata: Holothuroidea).



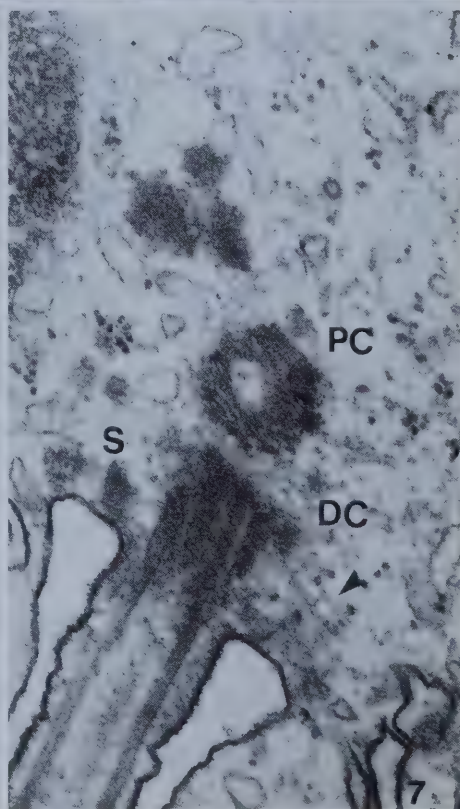
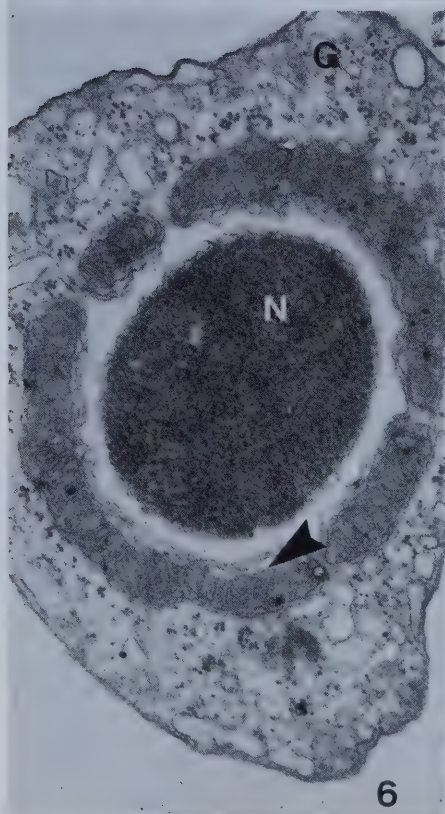
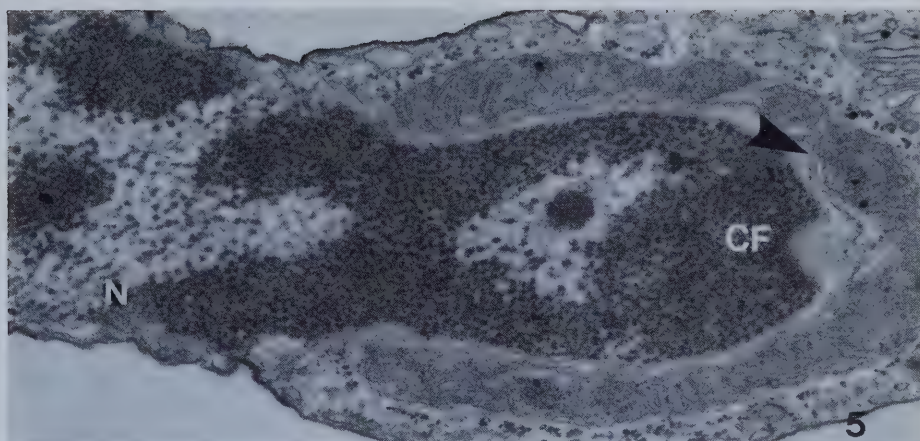
## PLATE I







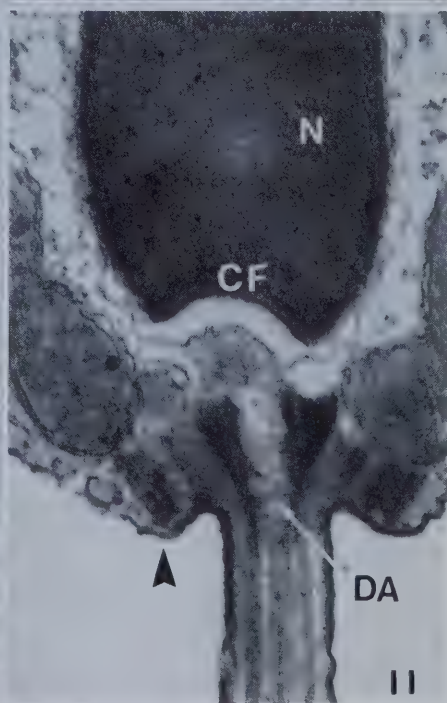
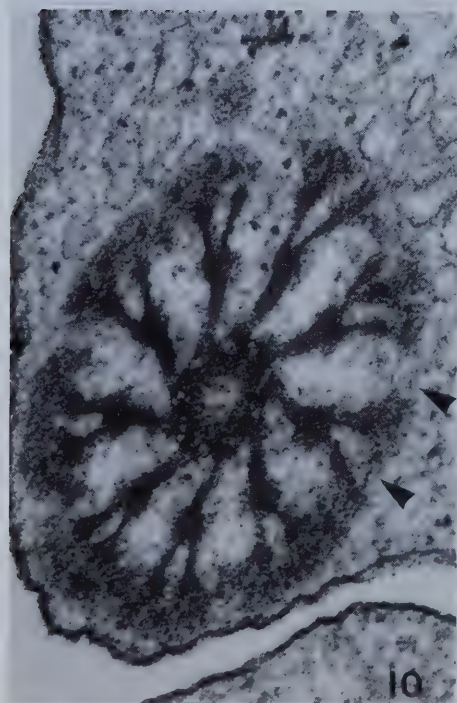
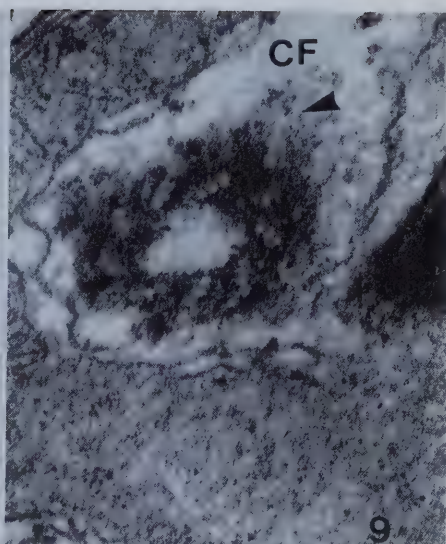
## PLATE II







## PLATE III





## PLATE IV

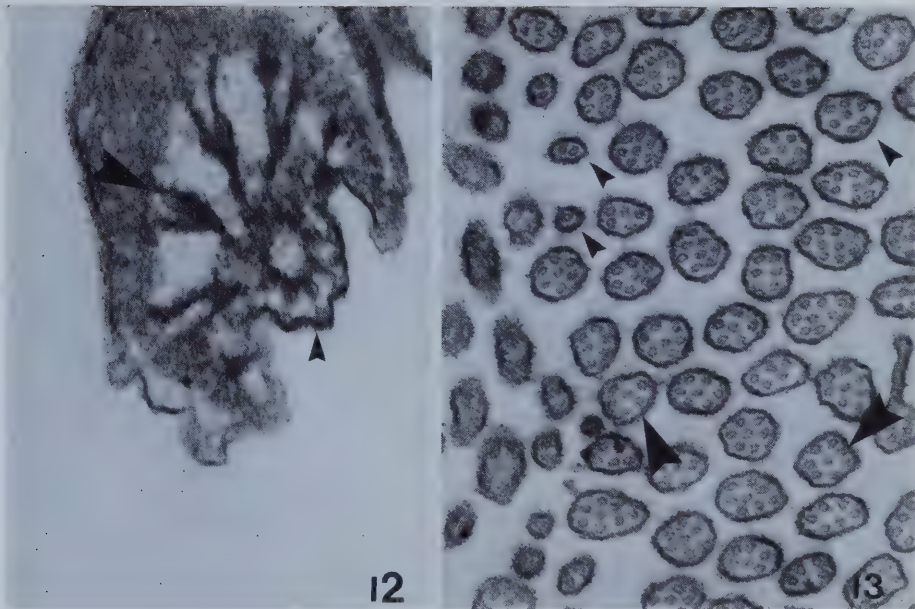


FIG. 3. Scanning electron micrograph of spermatozoon. A, acrosome; M, mitochondrion; N, nucleus. 30 000  $\times$ . FIG. 4. Longitudinal section of the acrosomal region. D, electron-dense disc; F, fibrous component of subacrosomal layer; GM, acrosomal membrane; N, nucleus; NM, nuclear membrane; PM, plasma membrane; SA, subacrosomal layer; VS, vesicular structure; black arrow, cup-shaped bands; white arrow, incomplete region of acrosomal granule membrane. 85 000  $\times$ .

FIG. 5. Longitudinal section showing relationship between the posterior nuclear region and the mitochondrial mass. CF, centriolar fossa; N, nucleus; arrow, single mitochondrion. 43 500  $\times$ . FIG. 6. Cross section of posterior nuclear region. G, glycogen-like particles; N, nucleus; arrow, mitochondrion. 45 500  $\times$ . FIG. 7. Longitudinal section of centriolar region. DC, distal centriole; PC, proximal centriole; S, satellite projections; arrow, microtubules. 78 500  $\times$ .

FIG. 8. Longitudinal section of centriolar region. P, dense projecting arm; CF, centriolar fossa; arrow, satellite projections of the distal centriole. 55 500  $\times$ . FIG. 9. Cross section through the triplets of the proximal centriole. CF, centriolar fossa; arrow, dense projecting arm. 132 500  $\times$ . FIG. 10. Cross section of the distal centriole and satellite projections. Arrows, microtubules associated with the tertiary fiber network. 85 000  $\times$ . FIG. 11. Longitudinal section through the posterior part of the nucleus and the distal centriole. CF, centriolar fossa; DA, arm of electron-dense material; N, posterior nuclear region; arrow, satellite projections. 58 500  $\times$ .

FIG. 12. Oblique section through satellite projections (large arrow) and Y-shaped membrane connectives (small arrow). 76 500  $\times$ . FIG. 13. Sections of spermatozoa tails showing reduced number of tubules (arrows). 71 500  $\times$ .





distal end as the number of tubules becomes reduced (Fig. 13) until only the plasma membrane is evident (Fig. 2).

### Discussion

*Cucumaria lubrica* is a small (5 cm when fully extended) dendrochirotid sea cucumber which occurs in swift water currents in great abundance (5000/m<sup>2</sup>) on subtidal rocky points of San Juan Island (Birkeland, personal communication). This species is known to brood its young between the ventral body surface and the substratum during the winter months, with the development being direct without a recognizable larval stage. It has been observed in the laboratory that after spawning, the sperm remain together in small bundles for an extended length of time before they are suspended (Engstrom, personal communication). The elongated sperm head may reflect a specific adaptation for the convenience of packaging of sperm bundles. This semipackaging of sperm is perhaps more advantageous in a brooding species of great population density, as it is well documented that sessile invertebrates of great densities have a tendency to form spermatophores. If this is so, one would expect the closely related species of supposedly similar methods of reproduction, *Cucumaria curata* and *Cucumaria pseudocurata*, to also have elongated sperm. But, on the other hand, if the sperm of *C. curata* and *C. pseudocurata* have spherical heads, it may indicate that they are different from *C. lubrica* in some important aspect of reproductive biology.

As far as the fine structure of the complete holothurian sperm is concerned, only two other species have been studied, *Leptosynapta clarki* (Atwood, unpublished observations<sup>1</sup>) and *Cucumaria miniata* (Fontaine, personal communication). All three species differ somewhat in anatomical detail. For example, the acrosome of *Leptosynapta* contains a circular membrane-bound granule with five centrally positioned electron-dense lamellae (Fig. 1). The acrosome of *C. miniata* contains an ill-defined granule totally different from either *C. lubrica* or *Leptosynapta*. The fibrous component observed in the subacrosomal layer of the *C. lubrica* sperm is ill defined and seldomly recognizable in *Leptosynapta*. Subacrosomal vesicular structures, consistently present in *C. lubrica* sperm, have never been observed in the spermatozoa of *Leptosynapta*. The most impressive difference in the

sperm morphology is the elongate, torpedo-shaped nucleus of *C. lubrica*. As has been described, the nucleus tapers toward the posterior end and is surrounded by the mitochondrial mass. On the other hand, the nuclei of *C. miniata* and *Leptosynapta* are spherical and lie anterior to the single mitochondrion.

### Acknowledgments

We thank Mr. Norman Engstrom for the collection of *Cucumaria* and Mr. G. D. Braybrook for his assistance on the scanning electron microscope. This study was supported by a National Research Council of Canada grant to F.-S. Chia.

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## Chapter V

SPERMATOGONIA, SPERMATOCYTES AND SPERMATIDS OF

*CUCUMARIA PSEUDOCURATA*



# SPERMATOGONIA, SPERMATOCYTES AND SPERMATIDS OF

## *CUCUMARIA PSEUDOCURATA*

### Introduction

During a recent comparative morphological study of echinoderm sperm it was observed that *Cucumaria pseudocurata* (Holothuroidea) produces a spermatozoon unique to the class, as well as to the phylum. Sperm shapes of two types have previously been reported in the class Holothuroidea: spherical (Atwood, 1974a; Krishnan and Dale, 1975; Fontaine and Lambert, unpublished manuscript) and cylindrical (Atwood and Chia, 1974). The spermatozoon of *C. pseudocurata* represents a third type: tabloid, i.e., elongated and dorso-ventrally compressed (Atwood, 1975). The ventral surface contains a medially situated acrosome with substructure differing from previously reported echinoderm species. The dorsal surface contains an extensive groove containing a striated rootlet-like structure extending from the centriolar region to slightly posterior to the acrosomal region. At the base of the elongated nucleus is a mitochondrion and a posteriorly projecting flagellum displaying a 9+3 tubular arrangement in the midtail region. The proximal and distal centrioles with their satellites lie perpendicular to one another within the mitochondrial mass. Finger-like cytoplasmic extensions project posteriorly from the midpiece (region containing mitochondrion, centrioles, satellites and striated rootlet), partially encompassing the basal region of the flagellum.

Subsequent examination of the spermatogenic process also revealed structural uniqueness, primarily in the morphogenesis of the acrosome and midpiece. To date, only one detailed published account is available on holothurian germinal cells less mature than sperm (Atwood, 1974b).



Accordingly, the present study describes the fine structure of spermatogonia, spermatocytes and spermatids in the sea cucumber, *Cucumaria pseudocurata*.

### Materials and Methods

*Cucumaria pseudocurata* were collected subtidally from September, 1973 through June, 1974 at Eagle Point, San Juan Island, Washington. Testes were removed from mature males and fixed in a glutaraldehyde-H<sub>2</sub>O<sub>2</sub> mixture prepared as follows. Twenty-five per cent glutaraldehyde (Fisher Scientific Company) was diluted to a 2.5% solution buffered to pH 7.6 with 0.34 M sodium chloride and 0.4 M phosphate buffer at room temperature. Thirty per cent H<sub>2</sub>O<sub>2</sub> (Fisher Scientific Company) was added with continuous stirring in the amount of ten drops per 50 ml of fixative. Fixation was for 2 1/2 hr at room temperature. A similar glutaraldehyde-H<sub>2</sub>O<sub>2</sub> technique has been reported by Peracchia and Mittler (1972). Tissues were then passed into 2.5% glutaraldehyde (with sodium chloride and phosphate buffer at pH 7.6) for 1 1/2 hr at room temperature, washed for 1 hr in phosphate buffer and post-fixed in 2% osmium tetroxide (in phosphate buffer) for 2 hr at room temperature.

Testicular tissues were then rinsed in 0.05 M maleic acid (pH 5.2) for 30 min with three changes and stained *en bloc* in saturated aqueous uranyl acetate for 30 min. Specimens were again rinsed in 0.05 M maleic acid, dehydrated, embedded in Araldite 502, and sectioned with a Porter-Blum-MT-2 ultramicrotome. Sections were stained with saturated aqueous uranyl acetate and 0.2% lead citrate and observed with a Philips EM 200. For light microscopy, Araldite sections were cut at 1  $\mu$  and stained



according to Richardson et al. (1960).

## Observations

### *General Description*

The germinal cells of the testes are arranged in a series of cell stages progressing from spermatogonia (normally in contact with the gonadal wall) to spermatocytes (located among and lumenally to the spermatogonia) to spermatids and spermatozoa (occurring centrally in the testicular lumen). Developing germinal cells are not separated into definite zones; however, they are generally more mature as they proceed from the gonadal wall toward the lumen. Six basic cell stages, determined by cell position, nuclear morphology and previous spermatogenic studies, are recognizable: (1) primary spermatogonia, (2) secondary spermatogonia, (3) early spermatocytes (preleptotene and leptotene stages), (4) late spermatocytes (zygotene, pachytene and diplotene stages), (5) spermatids and (6) spermatozoa.

The primary spermatogonium, roughly spherical in shape (Fig. 1), has a diameter of approximately  $8.7\ \mu$  and contains a roughly circular nucleus about  $5.5\ \mu$  in diameter containing one or two nucleoli, each having a diameter of  $1.8\ \mu$ . The nucleoplasm consists of a fine homogeneous matrix in which is suspended condensed chromatin distributed around the periphery of the nucleus and widely scattered throughout central regions. Connection-like junctions connect the spermatogonia. Cytoplasmic organelles include a Golgi complex, numerous ovoid mitochondria, limited rough endoplasmic reticulum, numerous free ribosomes, scattered polysomes and occasional microtubules. Membrane-bound





osmiophilic granules, lipid droplets and clear vesicles are normally present in the peripheral cytoplasm. Two centrioles, slightly angular to each other, lie in close proximity to the Golgi complex and nuclear membrane. In rare favorable sections the proximal centriole appears atypical in consisting of nine rows of five tubules each, instead of the usual three tubules. This is described further in the section on morphogenesis of the midpiece. Microvilli arise from spermatogonia, spermatocytes and spermatids with the greatest concentration being in the late spermatocyte stage.

Secondary spermatogonia (Fig. 2) presumably arise through mitotic divisions of the primary spermatogonia. This cell has an average diameter of  $7.0\ \mu$  with a nucleus of  $5.0\ \mu$  in diameter containing a single nucleolus with a diameter of  $1.1\ \mu$ . Nuclear changes include a smaller diameter, thickening of peripheral chromatin adjacent to the nuclear envelope and larger aggregations of centrally positioned chromatin. Cytoplasmic organelles and inclusions are the same as noted in the primary spermatogonia. Secondary spermatogonia are connected by connection-like junctions with no evidence of cytoplasmic bridges. While flagellar morphogenesis is occasionally initiated in this secondary stage (Fig. 2), this is infrequently encountered until the early spermatocyte.

Secondary spermatogonia differentiate into primary spermatocytes progressing through the preleptotene, leptotene, zygotene, pachytene and diplotene stages of the first meiotic prophase. Diplotene spermatocytes presumably give rise to secondary spermatocytes at the first meiotic division. Distinction between various spermatocyte stages was



very difficult in *C. pseudocurata*. For convenience, spermatocytes have been designated as early (preleptotene and leptotene stages) and late (zygotene, pachytene and diplotene stages). Secondary spermatocytes could not be conclusively identified.

Early spermatocytes (Fig. 3) have an average diameter of 5.4  $\mu$  with a nucleus measuring 3.7  $\mu$  in diameter containing a single nucleolus with a diameter of 0.6  $\mu$ . Chromatin condensation is greatly accelerated at this stage. Cytoplasmic organelles are the same as noted in the spermatogonia. The single Golgi complex is more extensive with an indication that proacrosomal granule formation has been initiated. The proximal and distal centrioles, in association with peripheral electron dense materials (presumably developing satellites), lie perpendicular to each other in the basal cytoplasm of the cell. The first signs of morphogenesis of the striated rootlet-like structure extending from the centriolar region are noted at this stage.

The late spermatocytes (Fig. 4) have an average diameter of 5.2  $\mu$  with a nucleus measuring 4.1  $\mu$  in diameter. No nucleolus is present. Condensed chromatin material is associated with distinct synaptonemal complexes. Cytoplasmic organelles are the same as described above. Microvilli arising from the cell surface appear to be more extensive at this stage. Proacrosomal granules have begun to coalesce to form an irregularly shaped acrosomal granule.

Early spermatids are irregularly circular (Fig. 5), measure about 3.4  $\mu$  and contain a nucleus with a diameter of 2.5  $\mu$ . No nucleolus or synaptonemal complexes are evident. Nuclear condensation has progressed from a particulate-fibrous state to a stage of coarse chromatin



granules interconnected by fine fibrous materials with extensive interchromatin spaces (Figs. 5, 6). Condensation proceeds at a faster rate in peripheral areas of the nucleus and gradually moves to the center. Spermatids contain microvilli and are joined together by intercellular cytoplasmic bridges. Several ovoid mitochondria (Fig. 5), a Golgi complex (Fig. 5), a basally derived flagellum (Fig. 5), and a densely staining chromatoid body (Fig. 12) are present in the cytoplasm. Dense satellite materials have accumulated in the vicinity of the distal (basal body) centriole (Fig. 5). In the basal cellular region, developing cytoplasmic extensions (folds) reach posteriorly encompassing the base of the flagellum (Fig. 5). An immature irregularly circular acrosomal granule is located in a slight nuclear depression on the future ventral surface of the cell (Fig. 5).

As the spermatid matures, nuclear interchromatin spaces are gradually obliterated by massive condensation of the dense chromatin granules (Figs. 9, 10, 11). The cell simultaneously elongates (no microtubules evident in peripheral cytoplasm) and compresses dorso-ventrally. The dorsal surface contains the developing striated rootlet-like structure located within a dorsal groove (morphogenesis described in detail below) and the ventral surface, a maturing acrosome situated in a nuclear depression (described below). Mitochondrial elements have transformed from several ovoid forms to a large single mass encompassing the centriolar-rootlet region. Cytoplasm is confined to the posterior cellular extensions and a narrow area surrounding the developing striated rootlet.





### *Morphogenesis of Acrósome*

Small proacrosomal vesicles are formed in the basal cytoplasm by the Golgi complex in the early spermatocyte stage. The majority of vesicles contain a fine reticular substance with a low affinity for stains with several vesicles being void of contents. In the early spermatid a large irregularly circular acrosomal granule measuring about  $0.6\ \mu \times 0.8\ \mu$  has formed, presumably from the coalescence of the smaller proacrosomal vesicles (Fig. 6). The contents which are of a highly sparse fine reticular nature are evenly distributed and not localized into definite zones as in *Leptosynapta clarki* (Holothuroidea) (Atwood, 1974a,b) and *Asterina pectinifera* (Asteroidea) (Dan and Sirakami, 1971). The surface of the granule facing away from the nucleus and Golgi complex is overlaid with a thin layer of dense material (Fig. 6). Small vesicles released from Golgi cisternae lie in close proximity to the adnuclear surface of the granule and possibly add to the contents of the granule (Fig. 6).

In a later stage, the granule is still somewhat irregularly circular, but has become reduced in size ( $0.5\ \mu \times 0.6\ \mu$ ) (Fig. 7). Granule contents have condensed from a sparse reticular state to a more electron dense particulate-fibrous nature (Fig. 7). The layer of dense material overlying the granule membrane on the surface opposite the Golgi cisternae has become increasingly more osmiophilic, with deposition of material being slightly greater on the inner surface of the membrane. As morphogenesis continues, granule materials become more condensed (Fig. 8). The dense material of the membrane is highly osmiophilic and obviously more concentrated on the inner surface (Fig. 8).



The granule has maintained its same relative position in relationship to the nucleus; i.e. the surface of the granule containing dense material is opposite to the nucleus.

As the spermatid matures, the acrosomal granule (presumably by migration) comes to lie between the plasma membrane and the nuclear envelope (Figs. 5, 9). The slight cup-shaped nuclear depression which houses the granule occurs on the side of the nucleus opposite the remaining cytoplasmic components (Golgi, mitochondria and flagellum). This side is destined to become the ventral surface of the mature spermatozoon. At this stage the granule appears to rotate within the nuclear depression ending up with the surface containing dense material being in an adnuclear position (Fig. 9). The granule is relatively circular, measuring about  $0.6\ \mu$  in diameter, and contains a homogeneous particulate material (Fig. 9). The dense material of the granule adnuclear membrane has become less osmiophilic, localized to the inside of the membrane and is of a fine particulate consistency (Fig. 9). Completely surrounding the granule is a periacrosomal layer of a homogeneous particulate-fibrous material more electron dense than that of the granule (Fig. 9). This layer, which does not appear to be a Golgi derivative, apparently arises from the cytoplasm which was sandwiched between the granule and the nuclear envelope at the time of granule implantation. A continuity between this material and the narrow zone of cytoplasm surrounding the spermatid is still evident immediately following implantation (Figs. 5, 9).

At a slightly later stage of morphogenesis (spermatid is elongating and becoming dorso-ventrally compressed) the anterior-posterior



surfaces of the nuclear depression flare out forming pockets in the surrounding nucleus. The anterior-posterior granule surfaces simultaneously bulge out as the granule sinks deeper into the nucleus (Fig. 10). The dense particulate material interior to the adnuclear surface of the granule membrane has now appeared to transform into an incomplete membrane-like structure (Fig. 10). The material of the periacrosomal layer remains homogeneous in nature but is becoming less osmiophilic.

In the late spermatid (nuclear elongation and chromatin condensation nearly completed) the acrosomal region is reaching maturity (Fig. 11). The anterior-posterior surfaces of the nuclear depression and granule continue to bulge out into the nucleus. The incomplete membrane-like structure is very distinct and extends from the anterior-posterior inner surfaces around the dorsal face of the granule. A space, consisting of sparse fibrous materials, has become evident between the granule material and the outer granule limiting membrane and extends ventrally from the positions where the inner incomplete membrane terminates (Fig. 11). The internal (dorsal) surface of the granule has correspondingly become slightly indented forming an inward depression (Fig. 11). In the completely mature acrosomal granule a small area of particulate-fibrous osmiophilic material occurs ventro-medial to this depression (Atwood, 1975). Two distinct regions of the previously homogeneous periacrosomal layer are becoming evident. First, dorso-medial to the granule is a particulate-fibrous material lodged within the granule depression. This material, surrounded by a space void of osmiophilic substances, is presumably the precursor of the acrosomal filament



(Fig. 11). The other region occurs ventral to the pockets on either side of the granule (between the granule and nuclear membranes) and is slightly more dense and granular than the remaining periacrosomal material. This area corresponds to the region within the granule where the inner incomplete membrane is lacking (Fig. 11).

### *Morphogenesis of Midpiece*

The region of the *C. pseudocurata* spermatozoon designated as the midpiece includes the proximal and distal centrioles, centriolar satellites, striated rootlet and dorsal rootlet groove. These components are encompassed by a large mitochondrion.

In the early spermatocyte the proximal centriole lies off center and perpendicular to the distal centriole (basal body) in the basal cytoplasm (Fig. 13). The distal centriole is typical in having nine rows of three tubules each, arranged in a pinwheel manner, whereas the proximal centriole is atypical displaying nine rows of what appears to be five tubules each. This configuration which is only occasionally discernible in spermatogonia and spermatocytes, is commonly noted in spermatids (Figs. 16, 19). In the mature spermatozoon, the number of tubules in the proximal centriole is difficult to determine due to the heavy deposition of dense materials associated with the striated rootlet and surrounding satellite. The first indication of development of the striated rootlet and associated structures is the equal deposition of dense materials between all nine rows of tubules in the wall of the proximal and distal centrioles (Fig. 13). Simultaneously, fine filamentous structures are forming at the proximal end of the distal centriole. They extend toward the center of the cell making contact with the





outer surface of the adjacent side of the proximal centriole. Small aggregations of osmiophilic substances are associated with these structures (Fig. 13). Projecting laterally from the wall of the distal centriole is a single club-shaped dense mass of material connected to the centriole by two dense regions (Fig. 13). This is the formative stage of the basal foot-like structure observed in late spermatocytes (Fig. 15). Posterior to this club-shaped mass, dense satellite materials are forming around the base of the distal centriole. The satellite material forms in close association to the developing basal foot and extends away from the centriole toward the plasma membrane (Fig. 13).

In the slightly later spermatocyte there is an increase in dense materials between the tubules in both centrioles with deposition occurring at a much greater rate in the proximal centriole (Fig. 14). At this stage, deposition in the proximal centriole is unequal with greater quantities of materials being formed in the vicinity of the tubules of the anterior (distal to the basal body) centriolar surface. This heavily osmiophilic material reaches away from the centriole in the form of a dense particulate arm (Fig. 14). In sections coinciding with the cross sectional axis of the proximal centriole there is a row of from three to five ill-defined circular densities parallel to the particulate arm. Presumably, the formation of these densities is initiated either directly by the proximal centriole or indirectly by the proximal centriole via the particulate arm (Fig. 14). The filamentous structures emanating from the end of the distal centriole have become more prominent and now display an ill-defined striated pattern



(Figs. 14, 15). The basal foot of the distal centriole, becoming more distinct in the later spermatocyte, clearly consists of a club-shaped cap connected to the centriolar wall by two dense regions. Fine dense fibers radiate from all surfaces of the foot into the surrounding cytoplasm with an exceptionally concentrated zone extending to the wall of the proximal centriole (Fig. 14). Satellite materials of the distal centriole have become more extensive and osmiophilic (Fig. 14).

In the late spermatocyte the dense projecting arm of the proximal centriole has transformed from a particulate to a fibrous state. The circular densities observed lying parallel to the arm are no longer present and evidently have been incorporated into the mass of the arm which has become more extensive (Fig. 15). The basal foot of the distal centriole has now reached maturity and consists of a dense cap separated from a stalk by a less osmiophilic space. The stalk is separated by another less osmiophilic space from the two dense connecting regions attached to the centriolar wall (Fig. 15). Fibers radiating from the basal foot are now restricted to the cap region and lie in a tight zone existing between the foot and the proximal centriole (Fig. 15).

By the early spermatid stage the basal foot and filamentous structures of the distal centriole have degenerated. The only remaining connection between the two centrioles is a narrow region of fibrous material existing from the end of the distal centriole to the basal surface of the proximal centriole (Fig. 16). This material increases in concentration by the intermediate spermatid and persists as a connection point between the centrioles throughout spermiogenesis (Figs. 17, 19, 20). In the early spermatid the proximal centriole has shifted slightly



laterally to lie directly above the distal centriole (Fig. 16). The fibrous arm of the proximal centriole (referred to as striated rootlet from this stage) has developed an ill-defined striation pattern (Fig. 16).

In the intermediate spermatid the striated rootlet has become very extensive, reaching deep into the cytoplasm (Figs. 17, 18). The rootlet consists of dense fibers, measuring 12  $\mu$ m in diameter, cross-striated by smaller fibers measuring about 9  $\mu$ m. The axial periodicity is about 39  $\mu$ m (Fig. 17). The rootlet originates at the proximal centriole as a single entity but bifurcates into several planes as it proceeds toward the nucleus (Fig. 17). As the rootlet elongates, a slight depression, which will become the dorsal rootlet groove in the late spermatid, develops in the posterior surface of the nucleus. Fine fibrous elements radiate from the projecting end of the rootlet into the nuclear depression (Fig. 17). Nuclear chromatin material is heavily condensed in this region and a conspicuous perinuclear space is evident between the outer and inner nuclear membranes (Figs. 17, 18). The nucleus is simultaneously compressing (Fig. 18, from top to bottom of micrograph) and elongating (Fig. 18, from left to right). A cross sectional view at a slightly later stage reveals that the rootlet elements contact only five of the nine rows of tubules of the proximal centriole (Fig. 20). Evidently, the tubules of the centriole have differential potentials for the formation of rootlet elements. The axial periodicity at this stage has increased to approximately 55  $\mu$ m (Fig. 20).

In the late spermatid the rootlet extends almost half the length of the cell with the axial periodicity being 55  $\mu$ m at the level of the





proximal centriole and 50  $\mu$  further anteriorly (same as observed in mature sperm) (Fig. 21). The terminal anterior end of the rootlet consists of 20—25 dense fibers tightly situated within the dorsal groove between the plasma and outer nuclear membranes. Satellite materials surrounding the proximal centriole are first evident in the late spermatid (Fig. 22). These materials encompass the base of the rootlet and are continuous with the satellite materials of the distal centriole (Fig. 22).

Basic cell shape first becomes altered in the intermediate spermatid where there is a corresponding compression and elongation of the nucleus (Figs. 5, 18). Compression takes place in the dorso-ventral plane, whereas elongation is along the anterior-posterior axis (Fig. 25A,B). In the late spermatid there is a gradual shift of remaining cytoplasm to the posterior dorsal region of the cell (Figs. 23, 24, 25C,D). This cytoplasmic shift is accompanied by an increase in nuclear compression and elongation. The rootlet groove which originated on the dorso-medial surface of the nucleus in the intermediate spermatid (Fig. 25B) elongates posteriorly as the cell matures (Fig. 25C,D). Through the cytoplasmic shifting process, the striated rootlet comes to lie parallel with and tightly situated within the groove (Fig. 25D).

### Discussion

The formation of acrosomal substructures in *C. pseudocurata* is very intriguing. Functional significance of these structures will remain obscure until fertilization experiments are completed. It is likewise interesting that a morphogenic polarity exists in the developing acrosome,



as evidenced by the facts: (1) the region of the acrosomal vesicle membrane surrounding the surface opposite the nucleus and Golgi complex always becomes overlaid with dense materials (precursor of the inner incomplete membrane of the mature acrosome), and (2) the acrosomal granule, following migration, appears to rotate within the nuclear depression ending up with the original complete surface lying adnuclear.

In the asteriod, *Asterina pectinifera*, the developing acrosomal granule maintains no specific orientation with respect to the nucleus; however, the surface on which dense materials are deposited is always at the side farther from the Golgi complex as in *C. pseudocurata* (Dan and Sirakami, 1971). In the holothurian, *Leptosynapta clarki*, the dense surface of the granule appears to remain in close association with the nuclear envelope throughout development with the granule undergoing no final rotation as in the present species (Atwood, 1974b).

It is generally believed in echinoderms that the acrosomal granule *migrates* from the basal cytoplasm region of spermatids to its final position at the sperm apex (Longo and Anderson, 1969; Atwood, 1974b). In a recent report on a holothurian, *Cucumaria frondosa*, Krishnan and Dale (1975) state that the acrosomal granule embeds in the nucleus and remains stationary as the remainder of the cytoplasm moves posteriorly beside the nucleus through 180°. In *C. pseudocurata* the granule embeds at a much later stage of development and distant to the region in which it is formed, thus suggesting that the granule migrates through the cytoplasm to its final location as in *Leptosynapta clarki*, *Cucumaria lubrica* (Atwood, 1974b) and echinoids (Longo and Anderson, 1969).



In *L. clarki*, *C. lubrica* as well as *C. pseudocurata*, the periacrosomal material appears to originate from the cytoplasm which is sandwiched between the acrosomal granule and nucleus at the time of granule implantation. Krishnan and Dale (1975) report in *C. frondosa* that the material originates directly from the acrosomal granule, since there is no cytoplasm between the nucleus and the acrosomal granule at the time it embeds. Evidently, significant morphogenic variations exist not only within the echinoderm phylum but also within the individual classes.

*C. pseudocurata* produces a spermatozoon which consists of a midpiece more complex than that observed in previously studied echinoderms. Functional reasons for this complexity remain obscure. In the early and late spermatocyte stages several structures develop which are presumably for anchorage and/or stability during the formative stages of the flagellum. These structures either disappear in later stages (basal foot, dense fibers radiating from basal foot), transform into mature structures (dense arm of proximal centriole, filamentous structures emanating from distal to proximal centriole) or persist throughout spermiogenesis (satellite materials of distal centriole).

An unique feature of the midpiece is the presence of a basal foot projecting from the distal centriole. This structure is almost identical in location and structure to the cup-shaped basal foot described for kinocilia of sensory cells in vertebrate lateral-line and equilibrium organs (Flock, 1965; Flock and Duvall, 1965; Thurm, 1968), retinal rods (Tokuyasu and Yamada, 1959), gill cilia of *Mytilus edulis* (lamellibranch) (Gibbons, 1961), statocyst sensory cells of *Helix pomatia* (Gastropoda) (Laverack, 1968) and anemone epithelial cells



(Peteya, personal communication). It has been postulated that the ciliary basal foot is a possible mechanosensitive structure instrumental in the control of directional movement of cilia (Thurm, 1968; Flock, 1971). Ciliated cells which contain basal feet exhibit a fixed directional ciliary beat which is related to the orientation of the feet. The questions that arise are why should a developing sperm cell be equipped with such a structure and for what reason does it degenerate? Three possibilities exist: (1) the basal foot is strictly an anchorage/stability structure only necessary during the formative stages of the flagellum and striated rootlet, (2) the foot is strictly a mechanosensitive device important in the initial movements of the flagellum or (3) the foot exhibits a combination of the above two functions.

Both the proximal and distal centrioles obviously have the capacity to organize filamentous cross striated materials of the midpiece (arm of proximal centriole and filamentous structures of distal centriole) as reported in mammalian sperm (Fawcett and Phillips, 1969). The filamentous materials observed in early and late spermatocytes passing from the distal to the proximal centriole appear relatively insignificant and degenerate by the early spermatid. It is possible, however, that these structures, under the influence of the distal centriole, act as a template or inducer for the development of the striated rootlet, which is basically a product of the proximal centriole. Striated rootlet-like structures observed in spermatozoa of various other organisms appear to be organized directly through the influence of the distal centriole (Fawcett and Phillips, 1969; Stanley, 1971; Fawcett, 1972; Mattei and Mattei, 1973).





Various views on the role of microtubules in the process of nuclear elongation have recently been published (Fawcett et al., 1971; Ferraguti and Lanzavecchia, 1971; Lanzavecchia and Donin, 1972). As in *Leptosynapta clarki* and *Cucumaria lubrica* (Atwood, 1974b), there is no evidence that microtubules play an active role in nuclear elongation in *C. pseudocurata*. Nuclear elongation in these species is probably due to internal condensation of the chromatin.

#### Acknowledgments

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PLATE 1

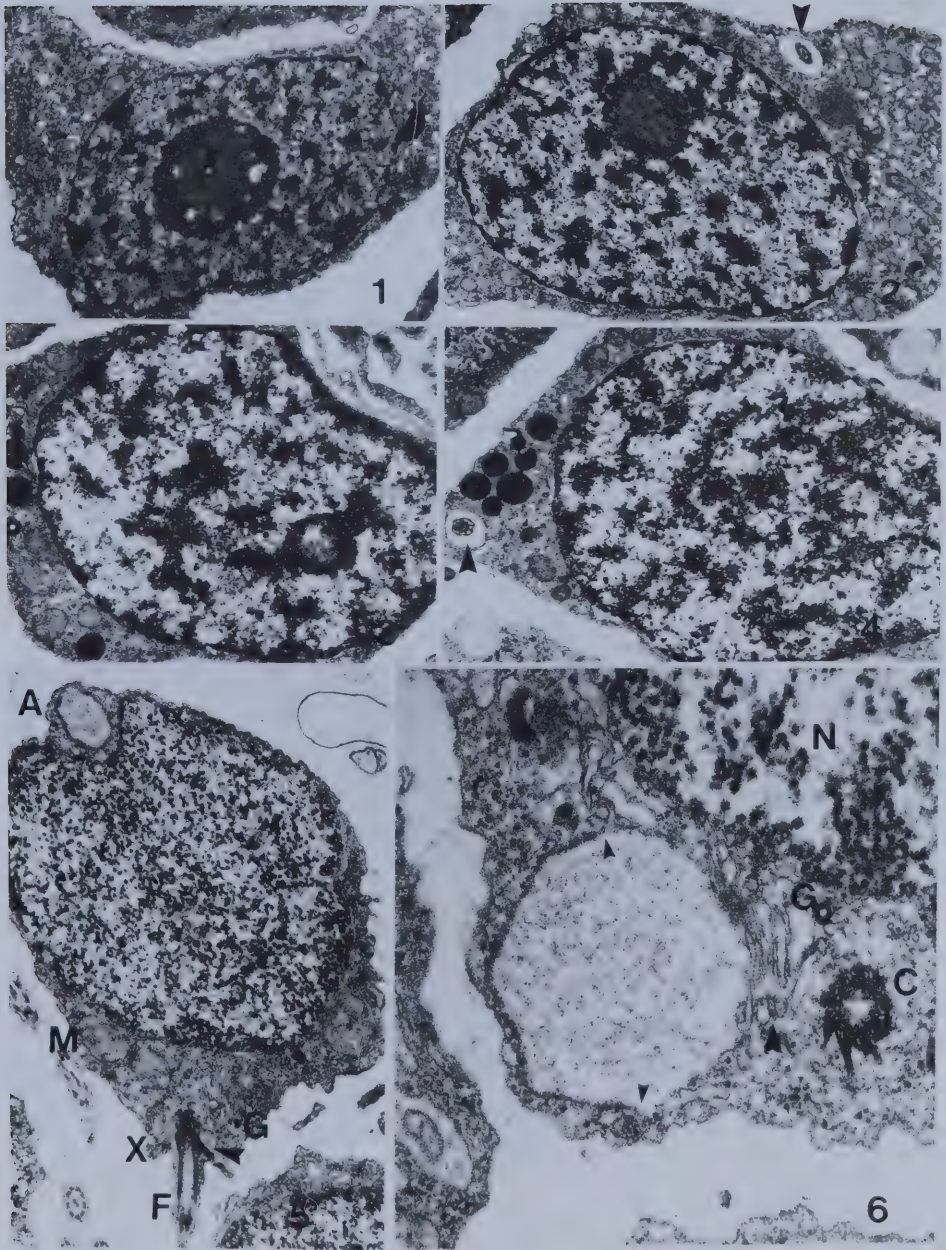
Figures 1—4. Early stages of spermatogenesis in *Cucumaria pseudo-curata*. The primary spermatogonia (fig. 1) normally lie in contact with the inner basal lamina of the haemal sinus and contain one or two large nucleoli. Interior to the primary spermatogonia lie the secondary spermatogonia (fig. 2), early spermatocytes (fig. 3), and late spermatocytes (fig. 4). Refer to text for complete description of cell stages. Figures 1, 2, X 11,600; figure 3, X 17,000; figure 4, X 13,100. Arrows, Flagellum.

Figure 5. Early spermatid. Note the irregularly circular shape, slight acrosomal nuclear depression and coarse nuclear chromatin granules, X 21,600.

A, Acrosomal region	M, Mitochondria
F, Flagellum	X, Cytoplasmic extensions
G, Golgi complex	Arrow, Satellite material of the distal centriole

Figure 6. Initial stage of acrosomal granule formation in early spermatid. Note the dense material overlying the region of the granule membrane opposite to the nucleus and Golgi complex. Granule contents are of a fine reticular nature, X 58,800.

C, Centriole	Arrows (small), Limits of dense material overlying complete membrane of acrosomal vesicle
G, Golgi complex	Arrow (large), Small vesicles
N, Nucleus	









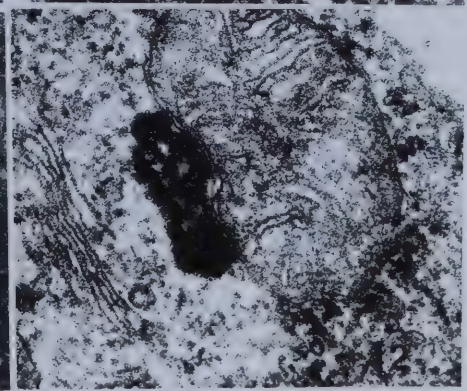
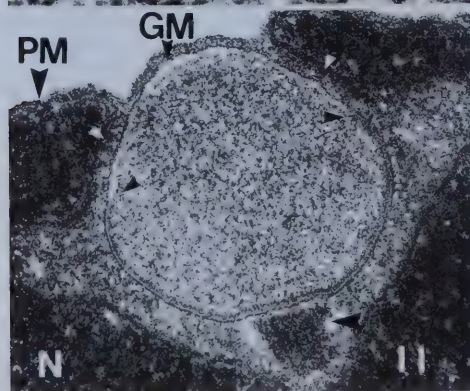
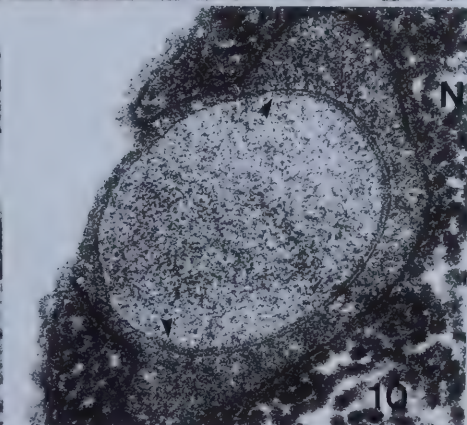
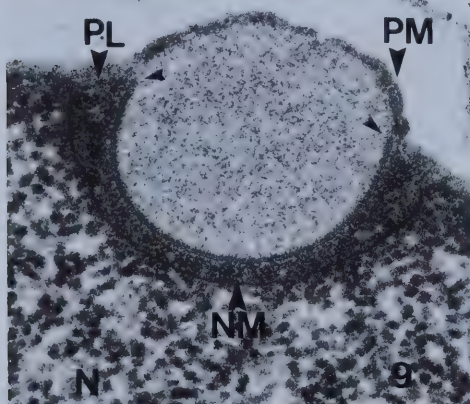
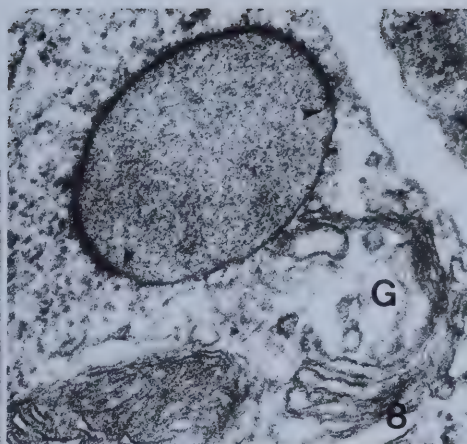
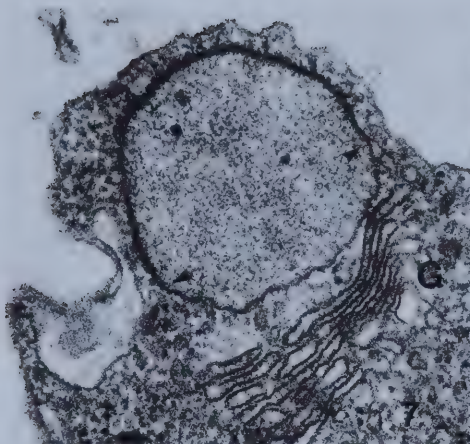
## PLATE 2

Figures 7—11. Sequential stages in the morphogenesis of the acrosome.

The granule becomes reduced in size and contains a particulate-fibrous material as the layer of dense material overlying the granule membrane becomes more osmiophilic (fig. 7). In a later stage (fig. 8), granule contents are condensing as deposition of dense material is localizing on the inner surface of the granule membrane. As the spermatid matures (figs. 9, 10), the granule comes to lie between the plasma and nuclear membranes in a nuclear depression. The granule rotates within the depression ending up with the surface containing dense material being adnuclear. Surrounding the granule is a periacrosomal layer of a homogeneous particulate-fibrous material. The dense particulate material interior to the granule membrane has transformed into an incomplete membrane-like structure. The anterior-posterior surfaces of the nuclear depression are beginning to flare out forming pockets in the surrounding nucleus. In the late spermatid (fig. 11) the acrosome is reaching maturity. Note the dense material of the periacrosomal layer lodged within the granule depression. Figure 7, X 66,150; figure 8, X 66,150; figure 9, X 61,600; figure 10, X 77,600; figure 11, X 87,300.

G, Golgi complex	Black arrows (small), Limited of
GM, Granule membrane	dense material (membrane-like
N, Nucleus	structure) interior to
NM, Nuclear membrane	granule membrane
PL, Periacrosomal layer	Black arrow (large), Particulate-
PM, Plasma membrane	fibrous material of the
	periacrosomal layer
	White arrows (small), Ventral
	dense, granular areas of the
	periacrosomal layer

Figure 12. Dense staining chromatoid body in close association with mitochondrion in late spermatocyte and early spermatid stages, X 73,500.







### PLATE 3

Figures 13—18. Stages in morphogenesis of the striated rootlet and associated structures of the middle piece. Refer to text for detailed description. Figures 13, 14, early spermatocyte; figure 15, late spermatocyte; figure 16, early spermatid; figures 17, 18, intermediate spermatid. Figure 13, X 67,900; figure 14, X 56,000; figure 15, X 58,800; figure 16, X 66,200; figure 17, X 50,400; figure 18, X 18,900.

- A, Arm of proximal centriole
- D, Dense fibrous materials radiating from the basal foot to the surface of the proximal centriole
- DC, Distal centriole
- F, Basal foot of distal centriole
- G, Dorsal rootlet groove (formative stage)
- N, Nucleus
- PC, Proximal centriole
- R, Striated rootlet
- S, Satellite material of distal centriole
- Black arrow (small), Circular densities parallel to arm of proximal centriole
- Black arrow (large), Dense connecting material between centrioles
- White arrow, Region of bifurcation of striated rootlet.



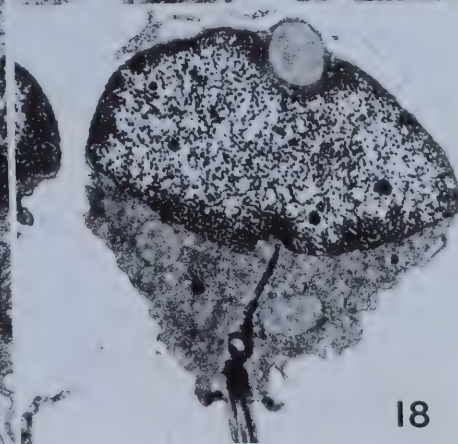
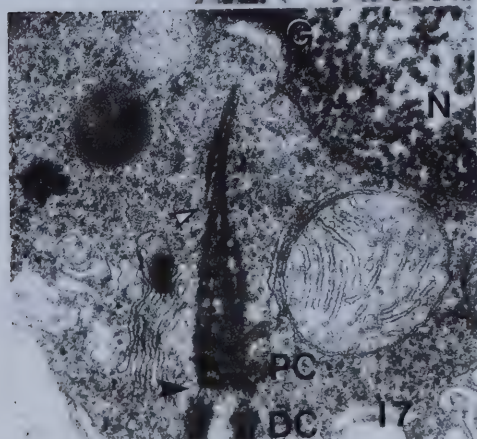
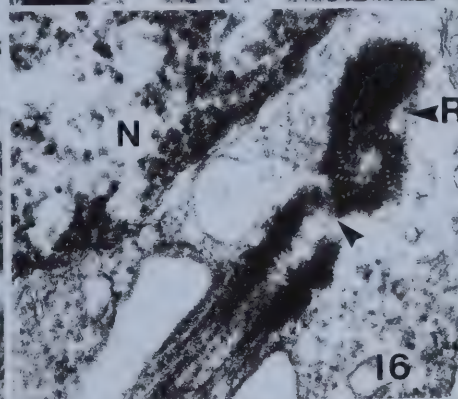
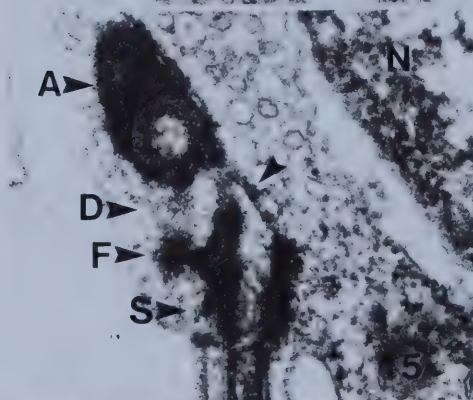
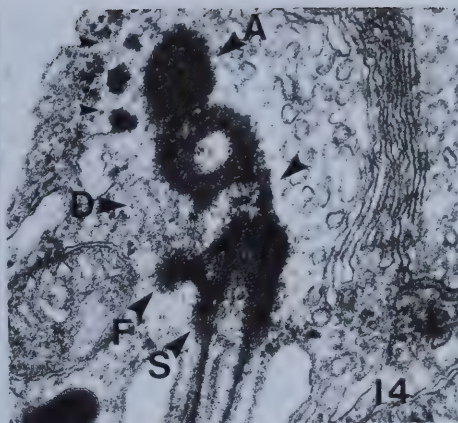
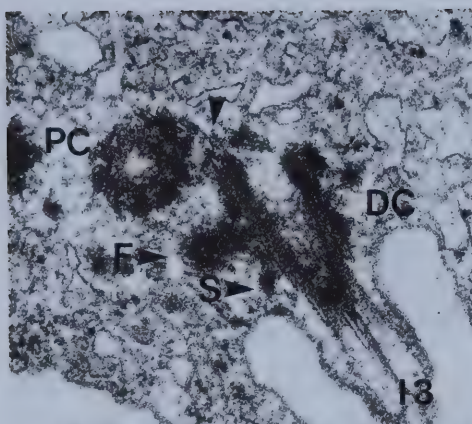








PLATE 4

Figure 19. Proximal centriole of an early spermatid. Note the nine rows of five tubules, X 147,000.

PC, Proximal centriole

White arrows, Tubules of one of the nine rows

Figure 20. Cross sectional view of the centriolar region of an intermediate spermatid. Note that the rootlet elements contact only five of the nine rows of tubules of the proximal centriole. The axial periodicity at this level of the rootlet is 55  $\mu$ , X 66,200.

Black arrows, Dense connecting material between centrioles.

Figure 21. Section through the longitudinal axis of the centriolar region of a late spermatid. Note the extensiveness of the striated rootlet. Axial periodicity is 55  $\mu$  at the level of the proximal centriole and 50  $\mu$  further anteriorly, X 39,200.

N, Nucleus

PC, Proximal centriole

S, Satellite material of the distal centriole

Figure 22. Cross sectional view of the centriolar region of a late spermatid. Note the satellite materials surrounding the base of the striated rootlet, proximal and distal centrioles, X 56,000.

DC, Distal centriole

PC, Proximal centriole

R, Striated rootlet

Black arrows, Satellite materials

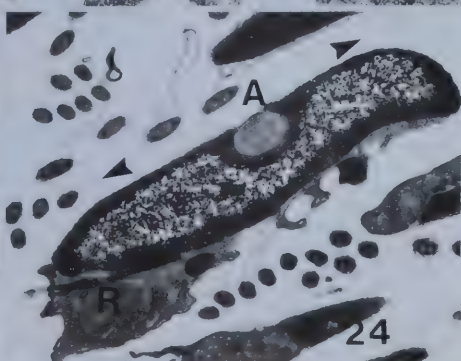
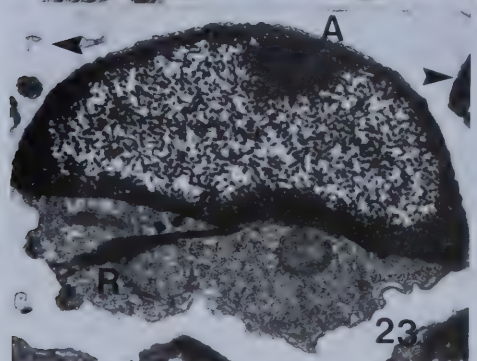
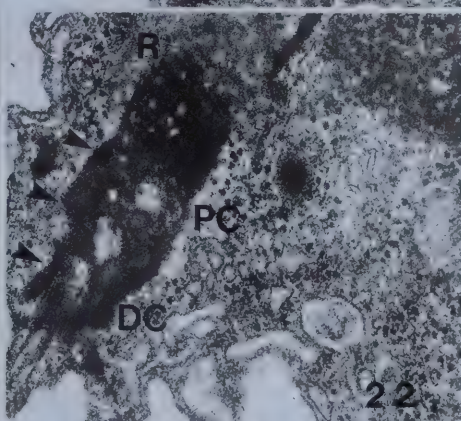
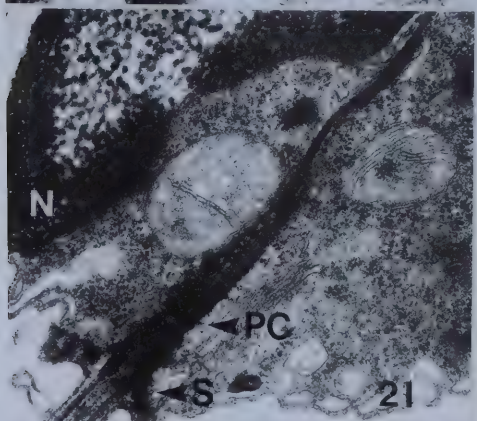
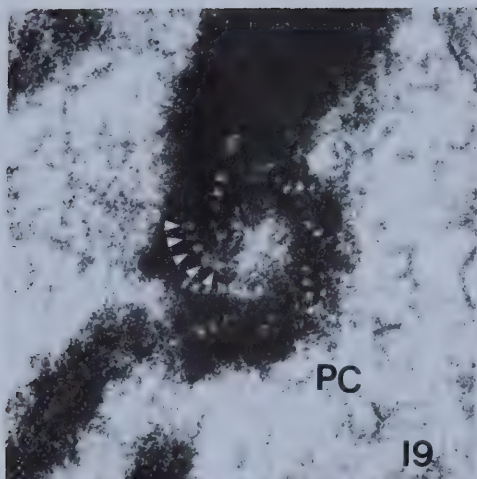
Figures 23, 24. Longitudinal sections through late spermatids.

Spermatid in figure 24 is at a slightly later stage demonstrating greater nuclear condensation and elongation. Refer to text and fig. 25 for detailed description of cell orientation and elongation pattern. Figure 23, X 18,900; figure 24, X 15,200.

A, Acrosomal region (ventral surface of spermatid)

R, Striated rootlet (dorsal surface of spermatid)

Black arrows, Direction of nuclear elongation (anterior-posterior axis)







## PLATE 5

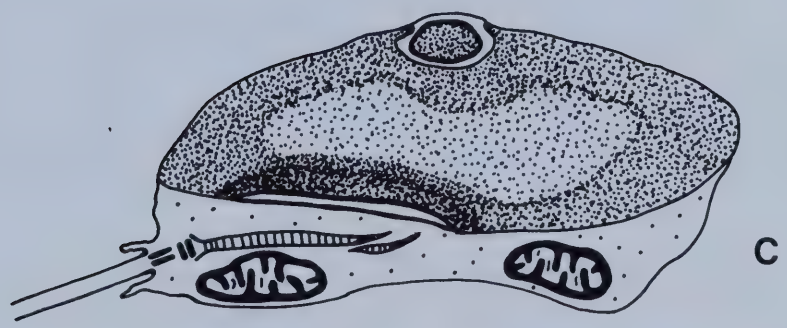
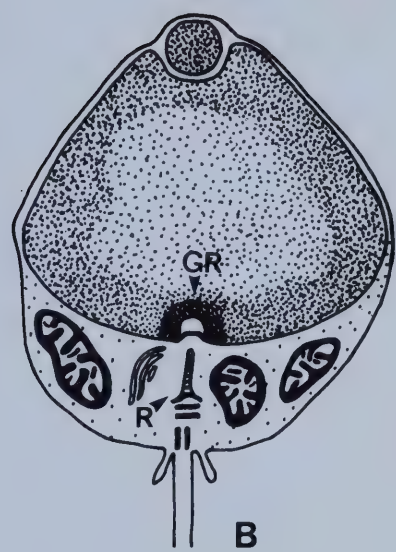
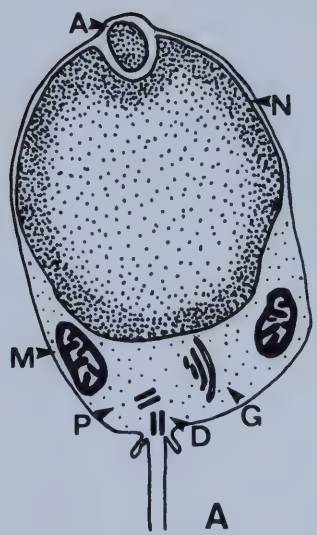
Figure 25. Diagrammatic representation of nuclear elongation and compression during spermiogenesis in *Cucumaria pseudocurata*.

Diagrams are through the longitudinal axis of an early spermatid (fig. 25A), intermediate spermatid (fig. 25B) and two late spermatids at different developmental stages (fig. 25C,D).

Orientation is such that the anterior-posterior axis runs from right to left on page and the dorso-ventral axis from bottom to top. Note the development of the dorsal striated rootlet groove in the intermediate spermatid stage (fig. 25B). As the nucleus elongates along the anterior-posterior axis, the dorsal groove elongates posteriorly from the level of the acrosome. There is a corresponding shift of remaining cytoplasm to the posterior dorsal region of the cell. The rootlet which develops in a plane perpendicular to the dorsal groove (fig. 25B) ends up in a parallel position in the late spermatid following cytoplasmic shift (fig. 25D).

- A, Acrosomal region
- D, Distal centriole
- G, Golgi complex
- GR, Dorsal striated rootlet groove
- M, Mitochondrial elements
- N, Nucleus
- P, Proximal centriole
- R, Striated rootlet









## Chapter VI

### SPERMATOZOON OF *CUCUMARIA PSEUDOCURATA*



## Fine Structure of an Elongated Dorso-ventrally Compressed Echinoderm (Holothuroidea) Spermatozoon

DAVID G. ATWOOD

Department of Zoology, University of Alberta, Edmonton, Alberta, Canada

**ABSTRACT** The spermatozoon of *Cucumaria pseudocurata* is unique among those of the echinoderms in that it is tabloid in shape, i.e., elongated and dorso-ventrally compressed. The sperm consists of a dorsal surface which contains an extensive striated rootlet-like structure located within a dorsal groove and a ventral surface which contains a medially situated acrosome. A single mitochondrion lies at the base of the nucleus. The flagellum is unusual in that a  $9 + 3$  tubular arrangement is observed in the mid-tail region.

The acrosome consists of an acrosomal granule bounded by a limiting membrane and a surrounding periacrosomal layer. The granule is irregular in shape with the anterior-posterior surfaces flaring out, forming pockets in the periacrosomal material. The ventral granule surface bulges forming a close association with the plasma membrane. The dorsal surface is indented. Ventral to the depression (within the granule) is a small area containing a particulate-fibrous material. To the inside of the granule limiting membrane there is a second membrane-like structure (incomplete) which extends from the anterior-posterior surfaces around the dorsal face of the granule. Dorso-medial to the granule the periacrosomal layer contains a particulate-fibrous region lodged within the granule depression. This material is presumably the precursor of the acrosomal filament.

Prominent cytoplasmic folds extend off from the basal flagellar region. The proximal and distal centrioles are situated perpendicular to one another within the mitochondrion. Centriolar satellite materials are associated with both centrioles. Toward the base of the tail the satellite of the distal centriole consists of nine radiating arms extending at an angle of  $45^\circ$  to the axis of the centriole. Each arm terminates in a dense thickening. The striated rootlet extends anteriorly from the distal centriole to just below the level of the acrosome.

Since the late nineteenth century numerous light microscopic observations have dealt with sperm morphology in the phylum Echinodermata (Field, 1893, 1895; Retzius, '05, '10; Dan, '50; Rothschild, '51; Dan, '52, '54; Colwin and Colwin, '55; Colwin and Colwin, '55, '56; Chia and Buchanan, '69). More recent and detailed anatomical accounts have reported on electron microscopy (Afzelius, '55; Dan, '60; Bernstein, '62; Dan et al., '64; Franklin, '65; Dan, '67; Hagiwara et al., '67; Anderson, '68; Austin, '68; Longo and Anderson, '69; Dan, '70; Fawcett, '70; Franklin, '70; Inoue et al., '70; Dan and Sirakami, '71; Afzelius, '72; Summers, '72; Atwood, '73; Longo, '73; Marshall and Luykx, '73; Atwood, '74; Atwood, in press; Atwood and

Chia, '74; Fontaine and Lambert, unpublished manuscript). Refer to Chia et al. (in press) for an up to date list of references on the light and electron microscopy of echinodermal sperm structure.

All spermatozoa of echinoderms examined have contained either a spherical (asteroidea, crinoidea, ophiuroidea, holothuroidea) or conical (echinoidea) shaped head positioned anteriorly to a short mid-piece consisting of a single uniformly shaped mitochondrion surrounding the centriolar region. A prominent acrosome always occurs at the apex of the nucleus. There is a general theory that sperm structure remains basically similar within each of the five echinoderm classes. However, recent observations indicate that extensive



morphological variations exist in the class Holothuroidea. Two basic sperm shapes have thus far been described in sea cucumbers. *Cucumaria miniata* (Fontaine and Lambert, unpublished manuscript) and *Leptosynapta clarki* (Atwood, '74) both contain a roughly spherical head, whereas that of *Cucumaria lubrica* (Atwood and Chia, '74) is of a cylindrical (torpedo) shape. Structural differences have also been noted in the acrosomal and centriolar-mitochondrial regions.

While examining sperm from various holothurian species it was noted that *Cucumaria pseudocurata* (an external brooder) produces a tabloid (compressed) spermatozoon, which differs from all echinoderm species previously reported. Closer examination revealed that the sperm had definite ventral and dorsal surfaces, an extensive striated rootlet-like structure lying in a dorsal groove, and a displaced ventro-medial acrosome. The fine structure of the mature spermatozoon of *Cucumaria pseudocurata* is here described.

#### MATERIALS AND METHODS

*Cucumaria pseudocurata* were collected subtidally in December, 1973 at Eagle Point, San Juan Island, Washington. Testes were removed from the mature males and fixed in a glutaraldehyde- $H_2O_2$  mixture prepared as follows. Twenty-five per cent glutaraldehyde (Fisher Scientific Company) is diluted to a 2.5% solution buffered to pH 7.6 with 0.34 M sodium chloride and 0.4 M phosphate buffer at room temperature. Thirty per cent  $H_2O_2$  (Fisher Scientific Company) is added with continuous stirring in the amount of 10 drops. Fixation was for two and one-half hours at room temperature. A similar glutaraldehyde- $H_2O_2$  technique has been reported by Peracchia and Mittler ('72). Tissues were then passed into 2.5% glutaraldehyde (pH 7.6) for one and one-half hours at room temperature, washed for one hour in buffer with four changes, and post-fixed in 2% osmium tetroxide (in buffer) for two hours at room temperature.

Testicular tissues were then rinsed in 0.05 M maleic acid (pH 5.2) for 30 minutes with three changes and stained *en bloc* in saturated aqueous uranyl acetate for 30 minutes. Specimens were again rinsed in 0.05 M maleic acid, dehydrated,

embedded in Araldite 502, and sectioned with a Porter-Blum-MT-2 ultramicrotome. Sections were stained with saturated aqueous uranyl acetate and 0.2% lead citrate and observed with a Philips EM 200. For light microscopy, Araldite sections were cut at  $1\ \mu$  and stained with Richardson's stain ('60).

For scanning electron microscopy, sperm were pipetted directly from the dissected testes into a solution of EDTA (sodium-tetraethylenediamine tetraacetate) (Fisher Scientific Co.) and filtered sea water (0.292 g EDTA added to 1000 ml sea water), which aids in removal of the excessive mucous coating. After washing three times in the above solution by centrifugation spermatozoa were fixed for 15 minutes at room temperature in a 2.5% glutaraldehyde solution buffered to pH 7.6 with 0.34 M sodium chloride and 0.4 M phosphate buffer. Sperm were then washed in buffer (0.4 M phosphate) and transferred to a 2% osmium tetroxide (in buffer) solution for 15 minutes at room temperature. Specimens were dehydrated in ascending concentrations of ethanol at 10-minute intervals and air dried. The dried spermatozoa were rotary shadowed with carbon then gold to 75–125 Å. A Cambridge S-4 Stereo Scan scanning electron microscope was used for the examination of specimens.

#### OBSERVATIONS AND RESULTS

The spermatozoon of *C. pseudocurata* is elongated (figs. 1, 2, 3, 4, 5) and dorso-ventrally compressed (figs. 3, 4). The sperm surface designated as ventral contains a large medially located acrosome (figs. 1, 4, 5) whereas the dorsal surface contains a prominent groove extending from the mitochondrial region anteriorly for approximately  $1.9\ \mu$  (fig. 2). Within the dorsal groove lie mitochondrial elements as well as an extensive striated rootlet-like structure (figs. 2, 4). The surfaces were labeled as ventral and dorsal based on the fact that in live preparations the sperm normally swim with the acrosomal region facing toward the substratum. The spermatozoon consists of a head measuring  $5.5\ \mu$  in length,  $1.2\ \mu$  in width and  $0.8\ \mu$  in depth; a mitochondrion at the base of the nucleus and a flagellum ranging from  $60\ \mu$  to  $70\ \mu$  (figs. 1, 3, 4). The sperm will be described according to the





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following regions: acrosome, nucleus, mitochondrion, centriolar complex, and flagellum.

*Acrosome*

The acrosomal region, encompassed by the plasma membrane, is very extensive in the *C. pseudocurata* spermatozoon (fig. 4). An acrosomal granule composed of a dense homogeneous particulate material and surrounded by a limiting membrane rests in a nuclear depression measuring about  $1.1\ \mu$  by  $0.6\ \mu$  (fig. 6). The granule is irregular in shape with the width (anterior-posterior axis) ( $730\ m\mu$ ) measuring greater than the depth (dorsal-ventral axis) ( $425\ m\mu$ ). The anterior-posterior surfaces flare out forming pockets in the surrounding periacrosomal material. The external (ventral) surface bulges out to form a close association with the overlying cell membrane (fig. 6). The internal (dorsal) surface is indented forming an inward depression in the granule. Ventral to this depression, within the granule, is a small area containing particulate-fibrous material of a greater electron density than the surrounding material. This material is normally somewhat circular in configuration and occurs in the middle of the inward depressed zone, flush with the granule membranes (fig. 6).

To the inside of the granule limiting membrane there appears a second membrane-like structure which extends from the anterior-posterior surfaces around the dorsal face of the granule (fig. 6). The membrane is incomplete and was never observed to extend more ventrally than the outer edges of the anterior-posterior granule pockets. A space, extending ventrally from the positions where the inner incomplete membrane stops, is evident between the granule material and the outer limiting membrane (fig. 6). This space, containing small quantities of fibrous material, becomes considerably greater around the ventral surface of the acrosome (figs. 4, 6).

Completely surrounding the granule is a periacrosomal layer which assumes the same shape as that of the granule. This layer consists of a particulate material slightly less electron dense than the granule (fig. 6). Areas of the periacrosomal layer ventral to the pockets (sandwiched

between the nuclear envelope and the granule membrane) are slightly more dense and granular than the remaining periacrosomal material (fig. 6). These zones could be correlated with the material composing the fibrous plate precursor of sea star acrosomes (Dan and Hagiwara, '67). These positions closely correspond to the regions within the granule where the inner incomplete granule membrane is absent. Dorsomedial to the granule the periacrosomal layer contains a distinct particulate-fibrous region lodged within the granule depression (fig. 6). This area, which is presumably the precursor of the acrosomal filament, is normally surrounded on all sides by a space void of electron dense materials.

From a surface view obtained with the scanning electron microscope it is noted that two areas normally protrude from the acrosomal region (fig. 5). The inner extended area is the ventral apex of the granule, whereas the peripheral ridge is presumably caused by the anterior-posterior granule pockets pressing ventrally against the plasma membrane. It is conceivable that these areas are over-emphasized due to the air drying procedure employed.

*Nucleus*

The nucleus, which is dorso-ventrally compressed, consists of chromatin condensed into coarse, electron dense granules arranged in irregular patterns (fig. 6). Nuclear vacuoles (noted in *Leptosynapta*, *Cucumaria lubrica* and *C. miniata*) are normally not observed, however, on several rare occasions small areas containing a fine reticular matrix and void of chromatin material were noted in the central regions of the nucleus. An extensive nuclear depression which houses the acrosome occurs on the ventral surface (fig. 4). This acrosomal nuclear indentation is medially located along the anterior-posterior axis of the sperm and extends almost completely through the depth of the nucleus (figs. 4, 6). Posteriorly, the nucleus is indented along the dorsal surface by a groove which contains a striated rootlet-like structure (figs. 2, 4). This dorsal groove extends from the centrioles (within the mitochondrial region) to just below the level of the acrosome. The nucleus, which is tapered



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at the anterior and posterior ends, reaches its maximum dimensions slightly posterior to the acrosomal depression.

*Mitochondrion*

A large single mitochondrion occurs immediately posterior to the nucleus (figs. 1, 2) and surrounds the proximal and distal centrioles, satellite projections and much of the rootlet-like structure (fig. 4). The mitochondrion is quite obvious on both the dorsal and ventral sperm surfaces with the greatest mass occurring on the dorsal side (figs. 1, 2, 4). Mitochondrial elements also lie within the striated rootlet groove (dorsal sperm surface) and extend for approximately two-thirds of its length (figs. 2, 4, 7) where they terminate posterior to the acrosomal region. Few mitochondrial granules are normally present. The mitochondrion appears to be orthodox as described by Hackenbrock ('66, '72).

Extensive cytoplasmic folds extend off from the mitochondrion (figs. 2, 4). These finger-like projections normally reach out posteriorly from the mid-piece and partially encompass the basal region of the flagellum (figs. 2, 4). On rare occasions these folds bend back onto the nuclear region of the ventral sperm surface (fig. 5). No mitochondrial elements were even observed within the cytoplasmic projections.

*Centriolar complex*

The proximal and distal centrioles (basal body), separated by a distance of  $0.1 \mu$ , are situated perpendicular to one another within the mitochondrion (figs. 4, 8, 9). The distal centriole contains the typical nine rows of three tubules, whereas the proximal centriole appears in rare favorable sections to consist of nine rows of five tubules. Due to the dense concentration of associated materials of the striated rootlet and satellite, this atypical configuration cannot be conclusively demonstrated. A narrow layer of cytoplasm, which extends anteriorly from the flagellar base, surrounds both centrioles (fig. 7).

Extending anteriorly from the distal centriole to just below the acrosomal region is an extensive striated rootlet-like structure (figs. 7, 8). The rootlet appears to originate at the proximal surface of the distal centriole and encompass the proximal centriole as it projects anteriorly (figs.

8, 9). Fine fibrous interconnections can be observed between elements of the rootlet and the outer surfaces of the proximal centriole (fig. 9). Slightly anterior to the proximal centriole the rootlet is made up of numerous dense anteriorly-posteriorly oriented fibers, measuring about  $12 \mu$  in diameter, cross striated by smaller diameter fibers measuring  $9 \mu$  (figs. 9, 10). The axial periodicity at this level is  $55 \mu$  (figs. 8, 9). From a longitudinal sectional view the rootlet appears to proceed toward the acrosome as a single entity (fig. 7). An oblique view reveals that the structure actually bifurcates with the anterior-posterior fibers separating into slightly different planes (fig. 8). Cross striations, which display an axial periodicity of about  $50 \mu$ , are not as distinct at this level (fig. 8). Further anteriorly the rootlet contains numerous interconnected fibers sandwiched between the outer nuclear membrane and the mitochondrial elements (fig. 11). Anterior to this level, the rootlet which consists of 20–25 dense fibers becomes more compact and is tightly situated within the dorsal groove between the plasma and outer nuclear membranes (fig. 12). The inner nuclear membrane is separated from the outer by a conspicuous perinuclear space (fig. 12). Near the termination level of the groove the fibers become smaller in diameter and reduced in number as the perinuclear space becomes correspondingly much greater in width (fig. 13).

Surrounding both centrioles and the posterior surface of the striated rootlet are dense satellite materials (figs. 4, 9). In the area of the proximal centriole the materials assume various shapes ranging from slender extensions protruding from the triplets to large dumbbell shaped spheres connected to the triplets by thin dense bridges (fig. 14). The satellite material appears at this level to have no patterned organization. The satellite material of the distal centriole, which is a continuation from the proximal centriole (fig. 9), is arranged in more of an ordered manner. Figures 15, 16, 17 and 18 are cross-sectional views of the distal centriolar satellite progressing from the proximal to the distal surfaces. Toward the base of the flagellum (distal end of centriole) the satellite materials assume the shape of nine



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radiating arms (fig. 18). Each arm radiates at an angle of approximately  $45^\circ$  to the axis of the centriole and terminates in a dense thickening. Dense fibrous material occurs between the satellite arms and is especially concentrated at the distal ends (fig. 18). Satellite elements lie in close association with surrounding mitochondrial membranes.

Below the level of the satellite projections (where the centriolar triplets become the doublets of the flagellum) Y-shaped membrane connectives extend from the A-tubules of the doublets to the scalloped plasmalemma (fig. 19A). A thin filament connects all nine A-tubules forming a nine-sided configuration. At this level no arms or spokes extend from the flagellar tubules. Small quantities of electron dense material are present between the central pair of tubules as well as in the area of the A-tubules of the peripheral doublets (fig. 19A).

#### Flagellum

*Cucumaria pseudocurata* sperm tails are  $60\ \mu$  to  $70\ \mu$  in length and about  $0.2\ \mu$  to  $0.3\ \mu$  in diameter. Flagellar substructure basically similar to that observed in this species has been described by Afzelius ('59) and Fawcett ('70). Just distal to the Y-shaped connectives the axonemal complex consists of the usual peripheral nine double tubules surrounding a central pair of single tubules with spokes and arms being present (fig. 19B). As in the area of the connectives, electron dense materials exist between the central tubules and in the region of the A-tubules of the peripheral doublets (fig. 19B). Distal to this region the diameter of the tail is reduced as spokes and dense materials become less conspicuous (fig. 19D). It is evident after examining over a thousand sections at a level slightly distal to figure 19D that a third central tubule exists between and slightly peripheral to the central pair of tubules, thus giving a  $9 + 3$  pattern to the flagellum (fig. 19C,E). The third tubule is normally in direct contact with the central pair (fig. 19E), however, appears to be separated by a small space from one or the other in many sections (fig. 19C). The  $9 + 3$  tubular arrangement was never observed in the basal (fig. 19B,D) or tip (fig. 19F) regions of the flagellum, indi-

cating that the third tubule originates and terminates along the midsection of the tail. In favorable sections a fourth tubule is present occurring as a mirror image of the third. This occurrence is, however, very rare and not convincingly shown. Toward the tip of the tail, which is extremely tapered, the tubules begin to terminate (fig. 19F) leaving only the plasma membrane at the most distal end.

#### DISCUSSION

Fine structural studies of holothurian species present great variation in acrosomal substructure. The acrosomal granule is limited by a membrane in *Leptosynapta clarki* (Atwood, '74) but not in *Cucumaria miniata* (Fontaine and Lambert, unpublished manuscript). In *Cucumaria lubrica* (Atwood and Chia, '74) a granule membrane is present, however, occasionally appears incomplete along the lateral surfaces. The granule in *Leptosynapta* contains four or five centrally located electron dense concentric lamellae, in *Cucumaria lubrica* a dense sphere anteriorly displaced and in *Cucumaria miniata* and *Thyone* (Summers et al., '71) a reticular zone of a sparse, coarse reticulum. Two electron dense, cup-shaped bands (membrane-like structures) have been noted in the posterior granule region in *Leptosynapta* and *Cucumaria lubrica*. These structures correspond closely to the primary membrane precursors (involved in the acrosomal reaction) of the sea star sperm (Dan and Hagiwara, '67). The incomplete membrane-like structure observed around the inside anterior-posterior and dorsal surfaces of the *Cucumaria pseudocurata* granule probably represents the primary as well as secondary membrane precursors noted in sea stars (Dan and Hagiwara, '67).

Several acrosomal structures of *C. pseudocurata* can, therefore, be equated to similar structures of echinoderm species in which fertilization has been examined. To determine functions of all the present structures it will be necessary to conduct detailed fertilization experimentation on this species.

Striated rootlet-like structures have been reported in a variety of mature and immature spermatozoa. In the mature spermatozoon of *Helix* (Mollusca) cross





striated rootlets are present with a periodicity of 400–500 Å (Anderson and Personne, '67). In *Pantodon buchholzi* (teleost) nine helical cross striated fibers (750–900 Å periodicity) run parallel to the nine mitochondria through the midpiece of the mature sperm (van Deurs, '73). A single rootlet having a periodicity of 500 Å has been observed in the mature spermatozoon of *Albula vulpes* (teleost) extending laterally from the distal centriole (Mattei and Mattei, '73).

In spermatids of *Squalus suckleyi* a rootlet-like structure (650 Å axial periodicity in early stages and 1350–1400 Å in later stages) extends from the distal centriole toward the nucleus and surrounds the proximal centriole (Stanley, '71). Evidently, an alteration of rootlet subunits occurs as development proceeds. *Xenopus laevis* spermatids and mature spermatozoa contain similar structures with a periodicity of 330 Å (Reed and Stanley, '72). Spermatocytes and early spermatids of *Cucumaria lubrica* and *Leptosynapta clarki* contain rootlet-like structures closely associated with both the proximal and distal centrioles. Axial periodicities are 620 Å in *Cucumaria* and 430 Å in *Leptosynapta*. These structures begin to disintegrate by the late spermatid stage and are completely absent from the mature spermatozoa (Atwood, in press).

The cross striated elements of the connecting piece within the neck region of mammalian spermatozoa (periodicity of 665 Å) could possibly be equated to the above mentioned rootlet-like structures (Fawcett and Phillips, '69). In mammalian species the striated columns originate mainly from the polymerization of filamentous material located in interstices in the wall of the distal centriole. This interstitial accumulation of material eventually results in distal disruption of the distal centriole in the mature sperm (Fawcett, '72). These cross striated elements become continuous with the outer fibers of the flagellum and continue anteriorly past the proximal centriole.

Morphogenesis of the cross striated elements in the *C. pseudocurata* sperm possibly represents a modification of the process observed in mammals, based on the following observations: (1) the distal centriole is intact in the mature sperm, (2)

striated elements do not appear to be continuous with flagellar components, (3) the elements appear to originate at the anterior surface rather than the sides of the distal centriole and (4) the proximal centriole (rather than the distal) appears to be more intimately involved with the cross striated elements. The genesis of this structure in *C. pseudocurata*, however, cannot be determined until a comprehensive study of spermatogenesis has been completed. The structure and location of the rootlet in *C. pseudocurata* suggests that it may function as an anchoring and/or stabilizing device for the distal centriole and flagellum.

In rare favorable sections of the mature sperm the satellite arms of the distal centriole extended beyond the terminal thickenings as two secondary fibers similar to the situation in *Leptosynapta* (Atwood, '74), *Cucumaria lubrica* (Atwood and Chia, '74) and *Cucumaria miniata* (Fontaine and Lambert, unpublished manuscript). This structural bifurcation was, however, very prominent in developing spermatocytes and spermatids of *C. pseudocurata*. Evidently, structural modifications in the satellite complex occur during morphogenesis of the sperm cell as noted in *Cucumaria miniata* and *Ophiopholis aculeata* (Fontaine and Lambert, unpublished manuscript).

Reger ('70) has likewise reported a 9 + 3 tubular arrangement in the sperm flagellum of *Pisaurina* (spider). In this species, however, the three central tubules were observed to originate at the center base of the distal centriole and run throughout the length of the tail.

All echinoderm spermatozoa examined to date have been of the "primitive type" (Franzen, '56). This type is characterized by a spherical (asteroidea, crinoidea, ophiuroidea, holothuroidea), conical (echinoidea) or cylindrical (holothuroidea) head anterior to a short midpiece containing a single mitochondrion surrounding the proximal and distal centrioles. These sperm contain a flagellum with a 9 + 2 tubular arrangement and a structurally complex acrosome mounted at the sperm apex. The primitive sperm type has been retained predominately in animal species which shed their gametes directly into the seawater and exhibit external fertilization.





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Since the presence of a globular primitive type head becomes a limiting factor as the viscosity of the medium increases the internally fertilizing sperm head is usually elongated and/or dorso-ventrally flattened with a filamentous, ovate, falciform, or ensiform shape. Structural adaptations in the motor apparatus to deal with this increased viscosity include (1) enlargement of the midpiece; i.e. greater quantity of mitochondrial elements in proportion to the remaining volume of the cell and (2) additional sets of fibers located in the flagellum (gastropods, cephalopods, cyclostomes, higher vertebrates).

The spermatozoon of *C. pseudocurata* has seemingly developed many of the characteristics (or at least modifications of these characteristics) observed in sperm specialized for internal fertilization. These particular adaptations have not appeared in any previously investigated echinoderm species, including those species which exhibit internal fertilization (Chia et al., in press).

*C. pseudocurata* broods the young externally between the ventral body surface and the substratum. The sperm are tightly packaged in dense strands of mucus and are released in the form of a modified spermatophore. Since embryos as young as the two-cell stage have been observed being brooded externally (Chia, personal communication) it is presumed that external fertilization occurs. The question that arises is for what reasons have these structural modifications evolved in a spermatozoon that fertilizes externally?

Several possibilities exist as answers to this question: (1) This species was originally an internal fertilizer which developed for some environmentally adaptive reason external fertilization, however, retained the original sperm conformation. (2) Sperm are released in close proximity to the female, migrate into the body (gonopore, oral cavity, epidermis) and internally fertilize the eggs. Immediately following fertilization the embryos are extruded from the ovary and brooded externally. (3) Sperm are released as packets in close proximity to a non-germinal cavity or surface fold (external or interior) on the female containing unfertilized eggs. These cavities contain a medium of high viscosity owing to body fluids or mucous secretions.

In the case of an internal cavity the fertilized eggs would immediately be released into the surrounding seawater. (4) Sperm structure is correlated with some particular aspect of the process of fertilization (surface configuration of egg, penetration of egg by sperm) rather than with the process of locomotion.

The first two possibilities appear unlikely since internal brooding following fertilization has definite protective advantages for the developing young. If this species has developed internal fertilization it seems remote that an internal brooding habit has not correspondingly been selected for. If it is assumed that sperm architecture is primarily based on properties of locomotion through media of varying viscosities, the third possibility is the most conceivable. Numerous holothurian species are known to brood young in non-germinal cavities or surface folds. In *Psolus granulatus* (Vaney, '25), *Psolidium incubans*, *Psolus punctatus* and *Psolus figulus* (Ekman, '25) the young are brooded in temporary ventral surface folds. In *Psolus ephippifer* (Théel, 1886) an anterior dorsal brooding chamber is formed by the interlocking of epidermal plates. Anterior brood chambers have also been noted in *Cucumaria coatsi* (Ekman, '25), *Psolus koehleri* and *Cucumaria joubini* (Vaney, '14, '25), *Cucumaria glacialis* (Mortensen, 1894) and *Cucumaria laevigata* (Lampert, 1889) contain ventral epidermal sacs extending into the body cavity. Several species brood their young in the body cavity (somatocoel), *Chiridota rotifera* (Clark, '10), *Synaptula hydriformis* (Clark, 1898), *Leptosynapta minuta* (Becher, '06) and *Thyone rubra* (Clark, '01). The spermatozoon structure of these species has never been examined, however, it is probable that modified sperm similar to *C. pseudocurata* are produced.

The female *Cucumaria pseudocurata* could possibly extrude unfertilized eggs into the sea, transfer them to the ventral surface with the use of tentacles and arrange with podia in superficial surface folds. The brooded egg masses are extremely sticky and surrounded by large quantities of mucus (field observations) similar to that described for *Cucumaria curata* (Smith, '62). The mucous secretions cover the entire body and evidently



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aid in the attachment of the eggs. The male being in close proximity to the female could spawn a modified spermatophore which would be carried into the mucus of the egg mass by water currents and fertilize the eggs externally. Structural modifications of the spermatozoon could, therefore, be correlated with the convenience of spermatophore packaging as well as locomotion through a medium of high viscosity similar to that observed in reproductive tracts of internally fertilizing species.

The fourth possibility is probable, however, unsolved until detailed fertilization experimentation has been completed.

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## PLATE 1

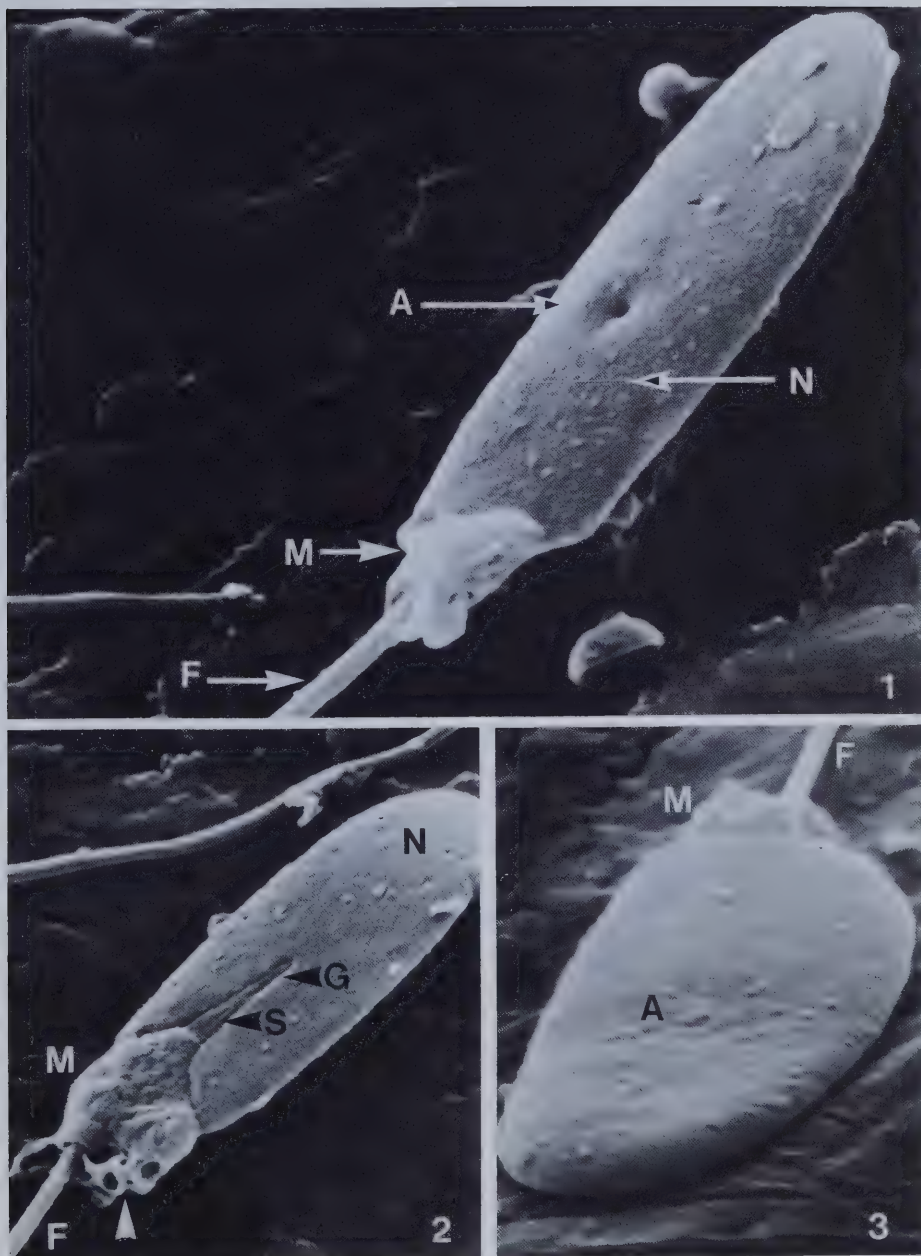
## EXPLANATION OF FIGURES

- 1 Scanning electron micrograph of ventral surface of the *C. pseudo-curata* spermatozoon. The acrosome is medial-ventrally situated between the sperm apex and the mitochondrion.  $\times 30,000$ .
- 2 Scanning electron micrograph of dorsal surface of sperm. The striated rootlet-like structure is situated within the dorsal groove which extends anteriorly from the mitochondrial region. Cytoplasmic projections extend posteriorly from the mitochondrion. Arrow, cytoplasmic extensions.  $\times 25,000$ .
- 3 Scanning electron micrograph of sperm apex and ventral surface. Note the dorso-ventral compression of the tabloid shaped spermatozoon.  $\times 40,000$ .

## Abbreviations

A, acrosome	M, mitochondrion
F, flagellum	N, nucleus
G, dorsal groove	S, striated rootlet







## PLATE 2

## EXPLANATION OF FIGURES

- 4 Longitudinal section of sperm with the anterior end at top of micrograph and posterior end at bottom. The ventral surface is at the left with dorsal surface facing toward the right. Note the dorso-ventral compression. Arrows, cytoplasmic extensions.  $\times 27,000$ .
- 5 Scanning electron micrograph of spermatozoan ventral surface. Two areas normally protrude from the acrosomal region. The inner area is the ventral apex of the acrosomal granule, whereas the surrounding peripheral ridge is the anterior-posterior granule pockets pressing ventrally against the plasma membrane. Note the cytoplasmic extensions folding anteriorly onto the nuclear region. Arrow, cytoplasmic extensions.  $\times 25,000$ .
- 6 Acrosomal region of the *C. pseudocurata* spermatozoon. Refer to text for detailed description. Black arrow (large), space between the granule material and the outer acrosomal membrane; black arrows (small), inner incomplete granule membrane; white arrow, particulate-fibrous material with the acrosomal granule.  $\times 97,000$ .

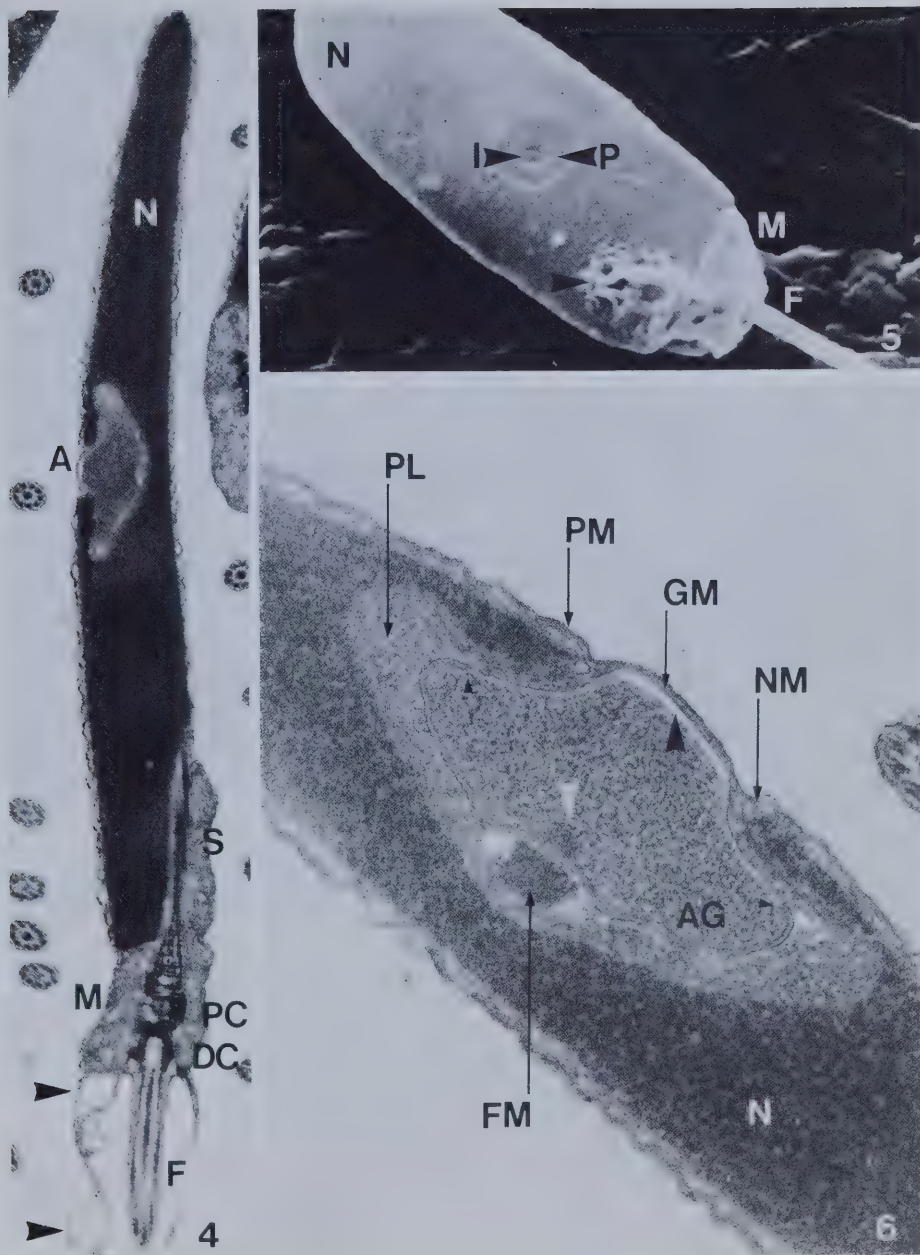
## Abbreviations

A, acrosome	M, mitochondrion
AG, acrosomal granule	N, nucleus
DC, distal centriole	NM, nuclear membrane
F, flagellum	P, peripheral acrosomal area
FM, fibrous material within the periacrosomal layer	PC, proximal centriole
GM, granule membrane	PL, periacrosomal layer
I, inner acrosomal area	PM, plasma membrane
	S, striated rootlet



TABLOID ECHINODERM SPERMATOZOON  
David G. Atwood

PLATE 2







## PLATE 3

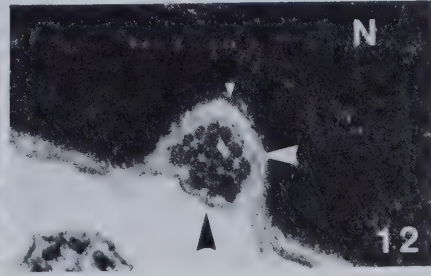
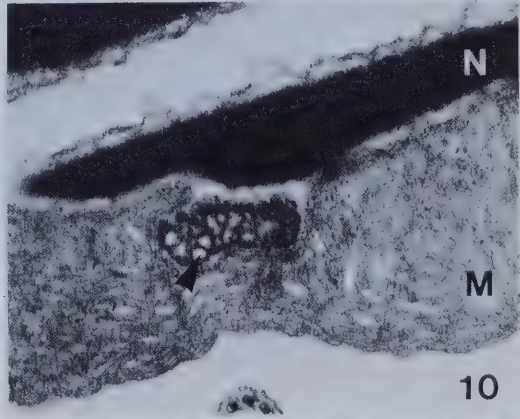
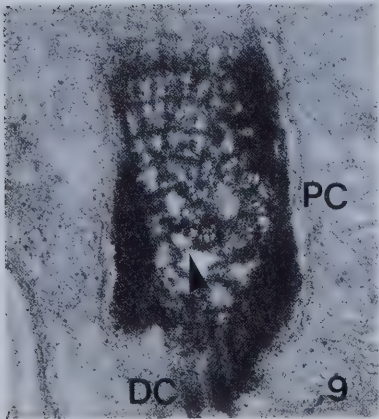
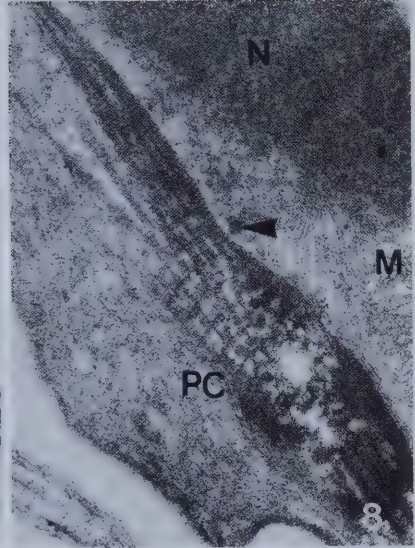
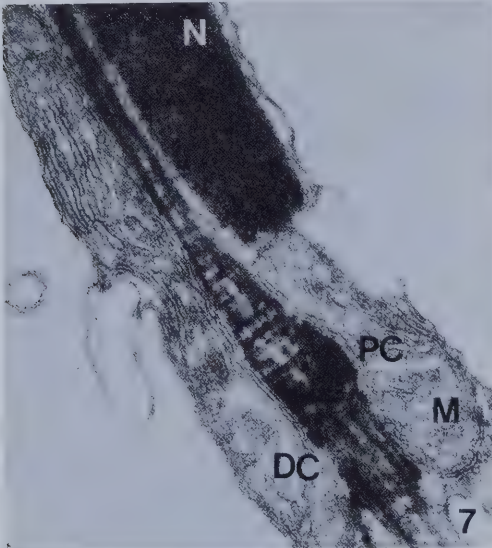
## EXPLANATION OF FIGURES

- 7 Longitudinal section of the centriolar-mitochondrial region. A narrow layer of cytoplasm surrounding both centrioles and the striated rootlet extends anteriorly from the flagellar base. Note the extensive rootlet extending toward the sperm apex from the distal centriolar region.  $\times 56,000$ .
- 8 Slightly oblique section through the centrioles and striated rootlet. Note the bifurcation of the anterior-posterior fibers of the rootlet at the level of the arrow. Arrow, level of bifurcation of rootlet elements.  $\times 61,500$ .
- 9 Sectional view of the centrioles and striated rootlet of the *C. pseudocurata* spermatozoon. Fine fibrous interconnections exist between elements of the rootlet and the proximal centriole. Note the dense satellite materials occurring peripherally to the centrioles. Arrow, fibrous interconnections.  $\times 79,500$ .
- 10-12 Cross sections of the striated rootlet progressing from the posterior to the anterior surface. Refer to text for detailed description. Black arrow (figure 10), cross striation fibers of the rootlet; black arrow (figure 12), plasma membrane; white arrow (large, figure 12), outer nuclear membrane; white arrow (small), inner nuclear membrane. Figure 10:  $\times 61,500$ ; figure 11:  $\times 51,000$ ; figure 12:  $\times 81,000$ .

## Abbreviations

N, nucleus	DC, distal centriole
PC, proximal centriole	M, mitochondrion







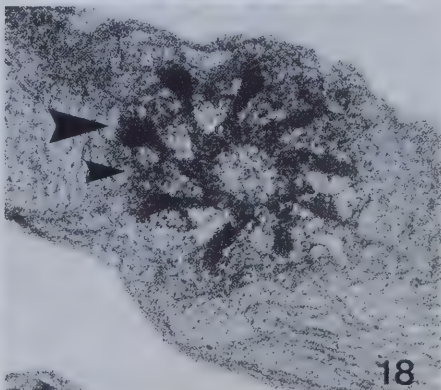
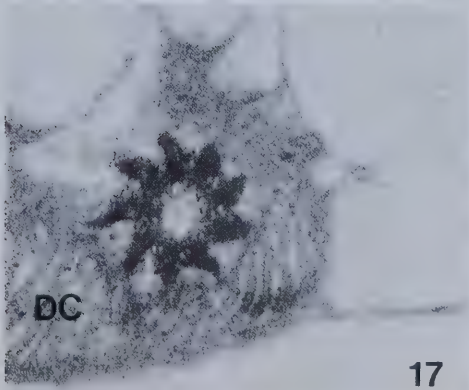
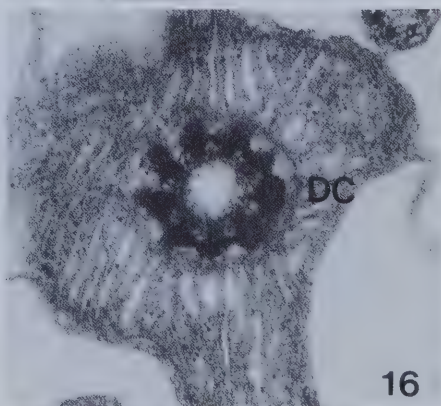
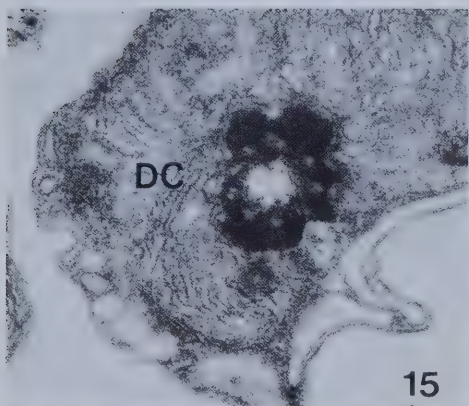
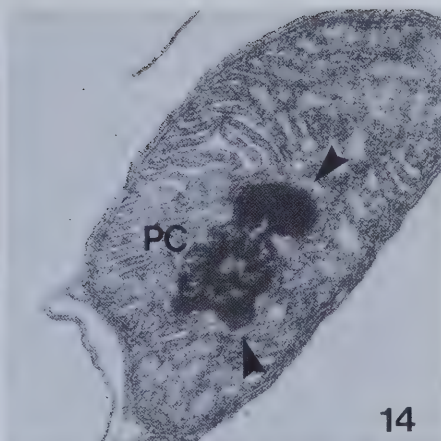
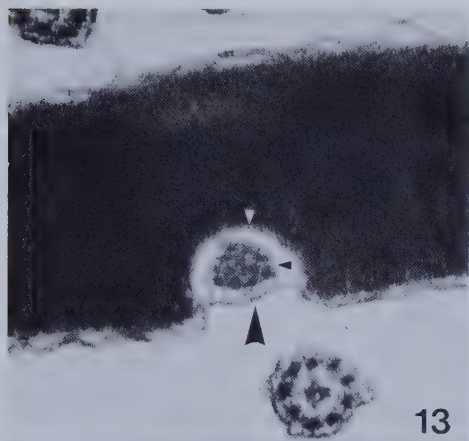
## PLATE 4

## EXPLANATION OF FIGURES

- 13 Cross section through the terminal anterior region of the striated rootlet. Refer to text for a description. Black arrow (large), plasma membrane; black arrow (small), outer nuclear membrane; white arrow, inner nuclear membrane.  $\times 73,500$ .
- 14 Cross sectional view of the proximal centriole and satellite projections. Note the variation in form and size of the satellite materials. PC, proximal centriole. Arrow, satellite projections.  $\times 59,000$ .
- 15-18 Cross sectional views progressing from the proximal to the distal surface of the distal centriole and centriolar satellite. Toward the base of the flagellum the satellite consists of nine radiating arms that terminate in dense thickenings. Fibrous material occurs between the arms. DC, distal centriole. Arrow (large), terminal dense thickenings; arrow (small), fibrous material between satellite arms. Figure 15:  $\times 67,200$ ; figures 16, 17:  $\times 87,300$ ; figure 18:  $\times 97,000$ .







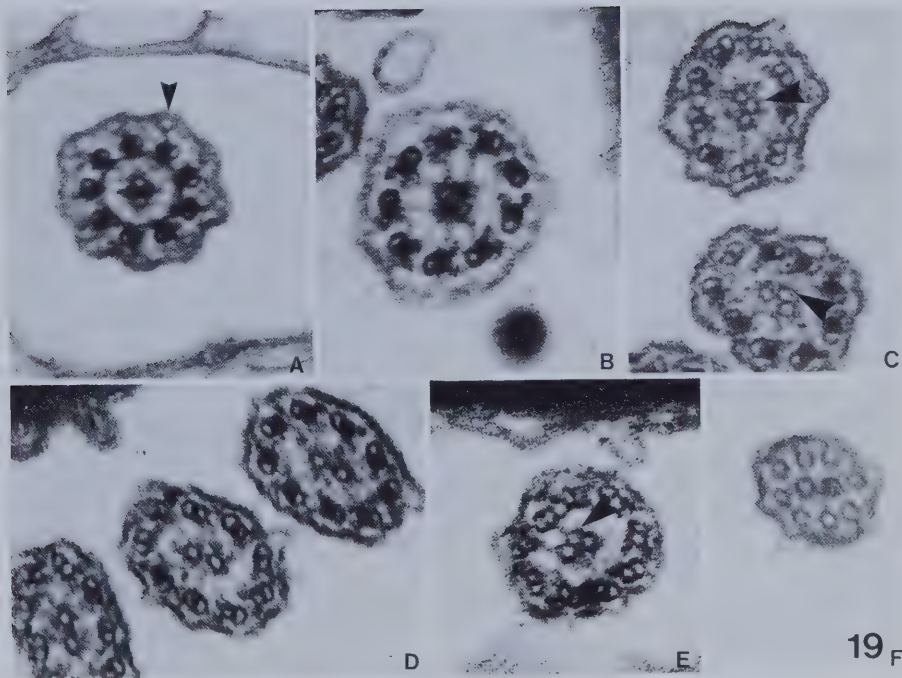


## PLATE 5

## EXPLANATION OF FIGURES

- 19A-F Cross sections through various regions of the flagellum of the *C. pseudocurata* spermatozoon. A. Basal region of flagellum which contains Y-shaped membrane connectives. Arrow, Y-shaped membrane connectives.  $\times 103,000$ . B. Region immediately posterior to the connectives, note the spokes, arms and dense materials associated with microtubules.  $\times 135,000$ . C. E. Mid-tail region revealing a  $9 + 3$  tubular pattern. Arrow, third central tubule presenting a  $9 + 3$  arrangement.  $\times 128,000$ . D. Mid-tail region showing the typical  $9 + 2$  arrangement.  $\times 127,000$ . F. Distal tip of flagellum with a reduced diameter and a reduction in the number of tubules.  $\times 154,800$ .







## Chapter VII

ACROSOMAL REACTION AND EGG INVESTMENTS IN  
*LEPTOSYNAPTA CLARKI*, *CUCUMARIA LÚBRICA*  
AND *CUCUMARIA PSEUDOCURATA*





## Introduction

Fol (1879) reported the existence of a long filament bridging fertilizing sea star sperm with the egg surface. This structure was later interpreted as an extension of the spermatozoon (Just, 1929). This sperm process, "acrosomal filament," and the cytological transformation of the sperm head, "acrosomal reaction," have been extensively studied in many echinoderm species (Dan, 1952, 1954a, 1956, 1960, 1967, 1970; Colwin and Colwin, 1955; Rothschild and Tyler, 1955; Afzelius, 1956a; Colwin and Colwin, 1956; Afzelius and Murray, 1957; Collier, 1959; Haino and Dan, 1961; Bernstein, 1962; Dan et al., 1962, 1964, 1972; Dan and Hagiwara, 1967; Franklin, 1970; Summers and Hylander, 1974). Dan (1952, 1954a, 1954b, 1956) has shown that the acrosomal reaction can be artificially induced by treatment with egg water, calcium-rich sea water, alkaline sea water, albumin sea water or contact with glass and collodion membrane surfaces. Dan (1952) demonstrated that the acrosomal reaction in sea urchins consists of two interrelated processes: 1) breakdown of the acrosomal membrane and the release of substances, possibly lytic enzymes, and 2) formation of a filamentous process from the acrosomal region. The actual dimensions and form of the process varies considerably among echinoderm species. It is generally accepted that in most echinoids the process is short ( $<0.7$  to  $6\ \mu$ ) and broad, while in holothuroids, asteroids, and ophiuroids it is extremely long (10 to  $75\ \mu$ ) and slender (Colwin and Colwin, 1956; Dan, 1956; Tyler and Tyler, 1966).



Ultrastructural studies have shown that all reported echinoderms produce sperm containing an apically located acrosome and, to the author's knowledge, all echinoderm sperm investigated can be artificially induced to discharge an acrosomal process. It has been shown that the sperm of *Cucumaria pseudocurata* (Atwood, 1975) deviates from the typical holothurian sperm model and conceivably deviates from the normal echinoderm acrosomal reaction. Sperm of *Leptosynapta clarki* (Atwood, 1974) and *Cucumaria lubrica* (Atwood and Chia, 1974), on the other hand, possess a typically situated acrosome. It is one aim of the present study to investigate with scanning electron microscopy this cellular phenomenon in these three holothurian species.

Ultrastructural studies of oocyte investment and cortical layers have been published for several echinoderm species (Afzelius, 1956b; Endo, 1961; Mercer and Wolpert, 1962; Franklin, 1965; Monroy, 1965; Tyler and Tyler, 1966; Lönning, 1967; Anderson, 1968; Kessel, 1968; Holland, 1971; Tegner and Epel, 1972; Summers and Hylander, 1974). Except for fragmentary reports in the classes Asteroidea (Monroy, 1965; Lönning, 1967), Ophiuroidea (Kessel, 1968), Holothuroidea (Lönning, 1967) and Crinoidea (Holland, 1971), all studies have dealt with Echinoidea species. No detailed comparable work is available for the class Holothuroidea.

Generally, the echinoderm female gamete is surrounded by a filamentous jelly envelope overlying a thin vitelline envelope which is separated from the egg cortex by a narrow perivitelline space (Austin, 1968). Endo (1961) and Anderson (1968) have shown that it is the vitelline envelope in echinoids which elevates from the oolemma during the cortical reaction to form the



fertilization membrane or activation calyx (Anderson, 1968). Irregularly spaced microvilli arise from the oolemma and extend through the perivitelline space into the vitelline envelope. Immediately interior to the oolemma is a layer of cortical granules (Harvey, 1911; Moser, 1939), spherical, membrane bound vesicles, varying in size and content between species (Tyler and Tyler, 1966; Lönning, 1967).

The second aim of the present research is to investigate the ultrastructure of the egg investment and cortical layers in the holothurian species *L. clarki*, *C. lubrica* and *C. pseudocurata* with scanning and transmission electron microscopy.

### Materials and Methods

*Leptosynapta clarki* were collected in October, 1974 at False Bay; *Cucumaria lubrica* in December, 1974 at Eagle Point; and *Cucumaria pseudocurata* in January, 1975 at Eagle Point, San Juan Island, Washington. Gametes were obtained by dissection of gonads from mature males and females. Female germinal cells of all three species were primary oocytes in the germinal vesicle stage and for convenience were referred to generally as eggs throughout this chapter.

The interaction of sperm and egg was not studied for *L. clarki* (an internal fertilizer) or *C. lubrica* (females not observed spawning). For *C. pseudocurata*, I was fortunate enough to obtain a recently inseminated brood of eggs from a laboratory holding aquarium.





For scanning electron microscopy of acrosomal reactions, 3 ml of dry sperm from each species is diluted in 40 ml of sea water at 8°C. One milliliter aliquots are removed from the diluted sperm solution and centrifuged for 3 min with a hand centrifuge. To artificially induce the acrosomal reaction, alkaline sea water (0.1 N  $\text{NH}_4\text{OH}$  added to sea water) at pH 9.8 is pipetted into the centrifuge tube containing the gently packed sperm pellet. After a period of 2 min, the sample is centrifuged and fixed in a 2.5% glutaraldehyde solution buffered to pH 7.6 with 0.34 M sodium chloride and 0.4 M phosphate buffer for 15 min. Sperm are then washed in buffer (0.4 M phosphate), pelleted by centrifugation and pipetted into Teflon "Flo-Thru Specimen Capsules" (Sargent-Welch Scientific Co.) containing Nuclepore membranes 25 mm in diameter with a 1  $\mu$  pore size (Sargent-Welch Scientific Co.). Specimens are post-fixed in 2% osmium tetroxide in 0.4 M phosphate buffer for 15 min at room temperature, dehydrated in ascending concentrations of ethanol and passed through ascending concentrations of amyl acetate to 100%. After critical point drying with carbon dioxide, the capsules are opened and samples on Nuclepore membranes from each capsule mounted on stubs with low-resistance contact cement. The material is coated with carbon then gold to a total thickness of 75 to 125 Å and examined with a Cambridge Stereo-Scan S-4 scanning electron microscope.

For scanning electron microscopy of investment layers, eggs are agitated from dissected ovaries and pipetted directly into a 2.5% glutaraldehyde solution (prepared as above) for 3 hr, followed by a wash in 0.4 M phosphate buffer. Eggs are secondarily fixed in 2% osmium tetroxide (prepared as above) for 1-1/2 hr and pipetted into specimen



capsules as previously described. Subsequent technique is as described for sperm acrosomal reactions. For scanning views of inner egg layers, individual eggs are placed on stubs with double-sided tape, ringed with low-resistant contact cement and fractured with razor blades. Specimens are then coated as usual.

For transmission electron microscopy of egg investment layers, eggs are pipetted into a 2.5% glutaraldehyde solution (prepared as above) for 3 hr at room temperature. Following a 1 hr wash in phosphate buffer with three changes, the eggs are post-fixed in 2% osmium tetroxide (prepared as above) for 2 hr at room temperature. Specimens are then dehydrated, embedded in araldite 502 and sectioned with a Porter-Blum-MT-2 ultramicrotome. Sections are stained with saturated aqueous uranyl acetate and 0.2% lead citrate and observed with a Philips EM 200. For light microscopy araldite sections are cut at 1  $\mu$  and stained according to Richardson et al. (1960).

## Results

### *Acrosomal Reaction*

The sperm of *Leptosynapta clarki* undergo a typical echinoderm acrosomal reaction, formation and protrusion of an acrosomal process, when treated with alkaline sea water. In the unreacted spermatozoon examined by scanning electron microscopy the acrosomal region normally appears slightly concave (Fig. 1). In many sperm treated for the full 2 min in alkaline sea water, the acrosomal region does not seem to be completely activated. The majority of these sperm are smaller in diameter than the fully reacted ones and instead of a lengthy process,



only a small bleb is apparent at the sperm apex (Fig. 2). The acrosomal process in the fully reacted sperm is relatively short (about 6  $\mu$  in length), slightly tapered at its apex and has a maximum width of approximately 0.2  $\mu$  (Fig. 3).

The fully reacted sperm of *Cucumaria lubrica* likewise displays a typical acrosomal process. Unlike that in *L. clarki* it does not taper distally and is only about 0.1  $\mu$  in width (Fig. 4). The actual length, which exceeds 35  $\mu$ , is impossible to determine due to the fragile nature of the process. Many semi-reacted spermatozoa are also evident in this species (Fig. 5). Whereas the apical bleb is similar to that formed in *L. clarki* no dimensional differences are noted between the heads of these sperm and those of the fully reacted sperm.

The sperm of *Cucumaria pseudocurata* differ from sperm of *L. clarki* and *C. lubrica* in that they are packaged in heavy mucous strands and are totally inactive for at least 5 to 7 hr subsequent to release. The sperm are arranged head to tail in long mucous bundles several hundred sperm in diameter. It is necessary for these bundles to be periodically agitated in sea water at 8°C for 10 to 12 hr before all sperm are motile.

Another species difference concerns the reactivity of the acrosomal region. After employing a variety of treatments (alkaline sea water at varying pH, calcium-rich sea water and albumin sea water) it was concluded that a typical acrosomal reaction involving the protrusion of an acrosomal process could not be artificially induced. Scanning electron microscopy revealed no structural changes in the acrosomal region following the above treatments. Subsequent work on naturally inseminated *C. pseudocurata*



eggs showed that the sperm in fact do undergo an acrosomal reaction which is unique to the echinoderms. It is still unclear why this reaction cannot be artificially induced.

A scanning view of the inseminated *C. pseudocurata* egg reveals that many sperm have attached to the outer egg envelope (detached sperm leave impressions on the egg envelope) (Fig. 6). As shown in figure 6, very few of these spermatozoa remain attached following preparatory procedures. Sperm detachment is possibly either due to technique procedures or it follows the same attachment-detachment sequence described in sea urchin eggs (Tegner and Epel, 1972). The spermatozoon attaches to the egg envelope on its side (lateral) rather than the typical *head-on* position (Figs. 7, 8). Evidently, the surface of the sperm (dorsal or ventral) that first contacts the egg is not critical, since 40% of the sperm observed were ventral side (acrosome containing surface) down and 60% ventral side up (50 eggs containing at least 20 attached sperm per egg were examined). Almost perfect impressions of the entire contacting sperm surface remains on the egg following detachment (Figs. 7, 9, 10, 11). Figure 11 shows the impression of the nuclear, mitochondrial, striated rootlet groove and flagellar regions of a detached sperm which was ventral side up.

Considering the varying configurations of attached sperm flagella (Figs. 7, 10) and the fact that the sperm contacting surface is not constant, it can be assumed that initial sperm orientation to the egg at the time of fertilization is random.

The acrosomal regions of all attached spermatozoa undergo structural modifications (Figs. 7, 8, 9, 10), possibly a modified acrosomal reaction,





in which no acrosomal process is formed. The acrosomal region appears hollowed out, as though the overlying plasma membrane has ruptured (Figs. 7, 9). In the majority of attached sperm, a tubular elevation extends from the center of the acrosomal region posteriorly toward the mitochondrion (Figs. 7, 8, 10). This elevation, which appears to lie under the sperm plasma membrane, cannot be explained until corresponding transmission electron microscopy has been completed.

The egg envelope in the vicinity of the attached sperm also undergoes structural changes, similar to chemical dissolution (Figs. 9, 10, 11). This dissolution is especially noticeable around the sperm body, is also evident along the entire length of the flagellum (Figs. 7, 9) and is obviously responsible for the impressions of detached spermatozoa.

#### *Egg Investment and Cortical Layers*

The *Leptosynapta clarki* egg is grayish-yellow in color and measures approximately 0.3 mm in diameter. Envelopes of eggs excised from ovarian tubules consist of the following layers progressing inwardly: 1) outer particulate-fibrous, 2) follicular cell, 3) dense laminate fibrous, 4) dense particulate, and 5) lucent particulate.

The outer particulate-fibrous layer (Figs. 12, 14), which varies in thickness depending on preparatory procedures, consists of a fine particulate material (Figs. 19, 20) and a meshwork of fibers, presumably of a collagenous origin (Figs. 14, 22). Directly beneath this is the layer of scattered follicular cells (Figs. 17, 19, 21) periodically joined together by desmosome-like structures (Fig. 20). These cells are closely applied to the dense laminate layer (Fig. 19) and send out highly branched (Fig. 18), thin (Figs. 19, 20) cytoplasmic processes.



The follicular cell nucleus normally contains one large nucleolus while the cytoplasm contains small ovoid mitochondria, numerous free ribosomes, membrane-bound dense granules and other typical cytoplasmic organelles (Figs. 19, 21).

Underlying the follicular cells is a dense laminate fibrous layer, consisting of fibrous elements in a stacked arrangement (Fig. 19), ranging in thickness from 0.1 to 0.4  $\mu$  (Figs. 14, 19, 20). In various regions around the egg this layer appears to bifurcate, forming a relatively large space containing fine dense particles and fibers as noted in the outer particulate-fibrous layer (Fig. 21). This laminate separation normally occurs in the vicinity of a follicular cell body (Fig. 21). Immediately interior to this layer are the two layers, dense particulate (Figs. 15, 19) and lucent particulate (Figs. 19, 20). No fibrous elements are noted in these regions. The two layers vary greatly in thickness ranging from 1.3 to 1.8  $\mu$  with the dense particulate normally being the thicker (Figs. 19, 20, 21).

The egg plasma membrane extends through the lucent particulate and into the base of the dense particulate layer as long, thin, microvilli (Figs. 13, 14, 15, 19, 20). The cortical layer directly underlying the plasmalemma contains numerous ribosomes, small ovoid mitochondria, large vesicular structures, endoplasmic reticulum and yolk granules (Figs. 16, 19). No typical echinoderm cortical granules (Harvey, 1911; Lönning, 1967) are apparent in the cortical layer.

The average *Cucumaria lubrica* egg is about 1.1 mm in diameter and green in color. Envelopes of oocytes excised from the ovaries consist of the following layers progressing inwardly:



1) follicular cell, 2) dense laminate fibrous, and 3) dense particulate. The scattered follicular cells contain a large nucleus with a single nucleolus and cytoplasm containing the usual organelles (Fig. 25). The cells send out long, thin cytoplasmic processes over the dense laminate fibrous layer (Fig. 26). The dense laminate fibrous layer (Figs. 23, 24) ranges in thickness from 0.1 to 0.8  $\mu$  (Figs. 25, 26) and is continuous, with no obvious separations as noted in *L. clarki*. Beneath this layer is a region of uniformly distributed electron-dense particles, the dense particulate layer (Figs. 24, 25, 26), whose thickness varies from about 4 to 7  $\mu$  (Figs. 25, 26).

The plasma membrane underlies this layer and extends slightly into it in the form of rather short, wide microvilli (Figs. 23, 24, 26). Electron-dense particles of the dense particulate layer are scarce at the bases of these cytoplasmic extensions (Fig. 27), possibly due to the physical barrier formed by the folded microvilli (Fig. 24) or fixation shrinkage. The egg cortical layer contains small ovoid mitochondria, few ribosomes, yolk granules, vesicular structures and no apparent cortical granules (Figs. 23, 25, 26).

The *Cucumaria pseudocurata* egg is orange and measures approximately 1 mm in diameter. The large excised eggs, which are tightly packed within the ovarian tubules (Figs. 28, 30), are externally surrounded by an extensively thick, dense laminate layer measuring about 8  $\mu$  (Figs. 28, 29, 30). This layer is composed of stacked, non-continuous laminae of relatively low electron-dense materials, each measuring about 0.2  $\mu$  in thickness (Figs. 30, 31). Immediately beneath these laminae is a continuous zone of material with the same structure





and density measuring about  $0.3 \mu$  in thickness (Figs. 29, 30, 31).

Directly beneath and in contact with the above layer is the plasma membrane of the egg (Fig. 31). No microvilli are evident. Underlying the plasmalemma is the egg cortical layer which consists of small ovoid mitochondria (Fig. 30), yolk granules (Figs. 28, 29, 30, 31), numerous vesicles occurring either singly or in groups of up to 20 (Figs. 29, 31) and no cortical granules. Also in this area are numerous membrane-bound electron-dense granules considerably smaller in diameter than the yolk granules (Figs. 28, 29, 30, 31). The granules vary in shape from spherical to elliptical and are of the same electron density as the yolk granules (Figs. 30, 31).

Figure 32 shows the outer layers of the inseminated *C. pseudocurata* egg. At the scanning level there appears to be no great structural changes induced by insemination, as observed in various echinoderm species. The small electron-dense granules are still present and are probably not involved in any type of cortical reaction (Fig. 32). Two minor morphological differences between the uninseminated and inseminated eggs are evident: 1) the dense laminate layer is slightly thicker after fertilization and 2) a layer with a fibrous consistency forms between the laminate layer and the cell membrane (Fig. 32).

## Discussion

In *Leptosynapta clarki* and *Cucumaria lubrica* two types of acrosomal reactivity are observed. The semi-reacted condition, where a small bleb is formed at the sperm apex, either represents a malformed process or a natural intermediate structural stage occurring prior to complete



acrosomal discharge. The latter is doubtful since the extension of the acrosomal process in other species is complete after a few seconds of exposure to the inductive solution (Dan, 1956). This raises the question of whether or not two types of sperm are produced in *L. clarki* and *C. lubrica*, one of which is non-functional. Dan (1954a) found that in the asteroids, *Asterina* and *Asterias*, the majority of the sperm which failed to react had smaller heads and likewise suggested the possibility of a type of non-functional sperm.

The sperm of *L. clarki* and *C. lubrica* undergo acrosomal reactions typical of echinoderms, whereas the reaction of the *C. pseudocurata* sperm is unique to the phylum. Subsequent sperm passage through egg investment layers is likewise unique to the phylum and tends to be similar to that reported in trematodes (Burton, 1967) and mammals (Austin, 1968; Bedford, 1972) which exhibit lateral fusion of gametes. The dissolution of the underlying egg investments could be postulated to be the result of egg lysins (trypsin-like enzymes) produced by the sperm. Presently, there are conflicting reports concerning the lytic activity of echinoderm sperm (Stambaugh and Buckley, 1972; Vacquier et al., 1972; Longo and Schuel, 1973; Summers and Hylander, 1974) with the majority of evidence disfavoring a trypsin-like enzyme sperm component. If in fact such enzymes are formed by the *C. pseudocurata* sperm, it is probable that they are not confined to the acrosomal region as in mammals (Stambaugh and Buckley, 1970), molluscs (Wada et al., 1956) and annelids (Colwin and Colwin, 1961a,b) but present along the entire length of the sperm.

Initial gamete contact in the holothurian *C. pseudocurata*



may be different from that described for other echinoderms. Summers and Hylander (1974) have suggested that echinoid gamete contact is a two-step process consisting of a binding between extracellular materials on the acrosomal process and the oolemma and then a membrane fusion between the acrosomal process tip and the oolemma. Aketa (1973) has postulated that a species-specific component is present on the apical end of the echinoid sperm which is complementary to a sperm-binding vitelline layer protein. These two molecules are possibly responsible for both initial species recognition and bonding of gametes. Summers and Hylander (1974) correspondingly suggest that such specific sperm molecules are contained within the acrosomal vesicle and are made available to the egg surface following the acrosomal reaction and that these molecules form a structural bond with the vitelline envelope prior to membrane fusion. Since observations indicate that no acrosomal process is formed in *C. pseudo-curata*, species recognition by the egg surface must be mediated through another structural region on the sperm. Conceivably, the egg-binding molecule, as suggested in echinoids, is localized in the acrosomal region and is discharged over the surface of the sperm at the time of sperm-egg contact. If this is the case, then the entire sperm surface rather than just the apical tip is responsible for species recognition. The mode of initial membrane contact and subsequent fusion is at the present unknown.

The physiological significance of the observed dissimilarity in the acrosomal reactions from spermatozoa of these three species remains to be elucidated. It is noted, however, that in *L. clarki* and *C. lubrica* the egg investment layers are quite similar as are the acrosomal reactions,



whereas, in *C. pseudocurata* both the egg investments and acrosomal reaction are quite different.

Lönning (1967) reported in *Cucumaria frondosa* that the jelly coat surrounding the oocyte is electron-dense and has an outer border with a layer of adhering follicle cells external to it and an inner border near the oolemma. The inner border is external to microvilli of the oolemma and is separated from the oolemma by a space void of jelly granular substances. Lönning referred to granule-like structures in the cortical region as cortical granules but, from the single micrograph, it appears as though they differ from cortical granules in asteroids and echinoids in density and substructure. There is no evidence that these structures are involved in the typical cortical reaction noted in echinoids. Substructure of the investment layers in *Cucumaria frondosa* due to poor fixation and resolution cannot be adequately compared to the substructure of the investment layers in this study.

Preliminary observations indicate that the outer particulate-fibrous layer of the *L. clarki* egg comes from the haemal sinus of the ovarian wall. The particulate as well as the collagenous-like fibrous component of this layer are characteristically noted in the ovarian haemal sinus in the earlier stages of oogenesis (Atwood, 1973). It appears as though developing oocytes pass through the germinal epithelium of the ovary into the haemal sinus at an early stage of development, presumably for nutritive reasons. The manner of release of the mature oocyte from the sinus is presently unknown.

Present observations indicate that significant species variation occurs in the substructure of holothurian egg surfaces. The egg investments of *L. clarki* and *C. lubrica* are similar, whereas that of *C. pseudocurata*





is different. The external layers of the *C. pseudocurata* egg are structurally dissimilar to the above two species in that: 1) the follicular cell layer is absent, 2) the laminate layer is very extensive and contains no fibrous component, 3) the dense particulate layer is absent, 4) microvilli of the plasma membrane are not evident, and 5) the egg cortical layer is vesiculated and contains small electron-dense granules different in morphology from the yolk granules. The physiological significance of these structural variations cannot be determined until the fine structure of fertilization has been described.

It is interesting, that the egg envelope in *C. pseudocurata* consists of only one homogeneous, extremely dense layer rather than the several different layers observed in *L. clarki* and *C. lubrica*. This single dense layer could possibly present a greater barrier to advancing sperm than the investments of the other two species. Perhaps a typical echinoderm acrosomal process (one without accompanying enzymatic activity) could not penetrate such an investment, whereas a sperm that does possess such activity could. Until research is completed on the biochemical properties of the acrosomal region, physical properties of acrosomal process and chemical properties of egg investments in many holothurian species, this problem will remain unsolved.

Investment layers of the three species studied in the present research surround pre-spawned primary oocytes in the germinal vesicle stage. It is conceivable that structural changes could occur in the investments following natural spawning. In the case of *L. clarki*, which is an internal fertilizer, mature eggs are retained within ovarian tubules and are not freely spawned to the outside. There is no doubt that the outer follicular cell layer remains intact during fertilization since it per-



sists through the early developmental stages of the embryo. In *C. pseudocurata*, naturally spawned eggs have been examined and no structural differences noted. In the case of *C. lubrica*, mature naturally spawned eggs have not been investigated. It is possible that in this species the outer follicular cell layer is shed prior to spawning.

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PLATE 1

Figure 1. Scanning electron micrograph (SEM) of the unreacted *Leptosynapta clarki* spermatozoon, X 36,000.

Figure 2. SEM of semi-reacted *L. clarki* sperm. Note: small bleb at sperm apex (arrow), X 27,000.

Figure 3. SEM of reacted *L. clarki* spermatozoon. Note: acrosomal process (arrow), X 18,000.

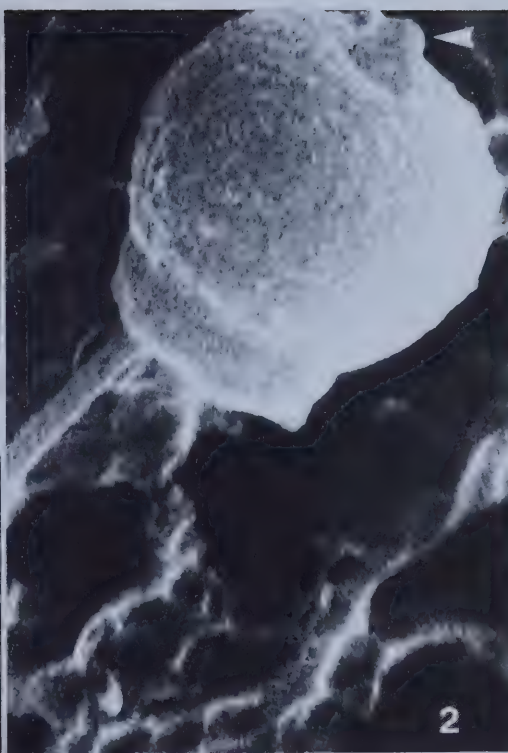
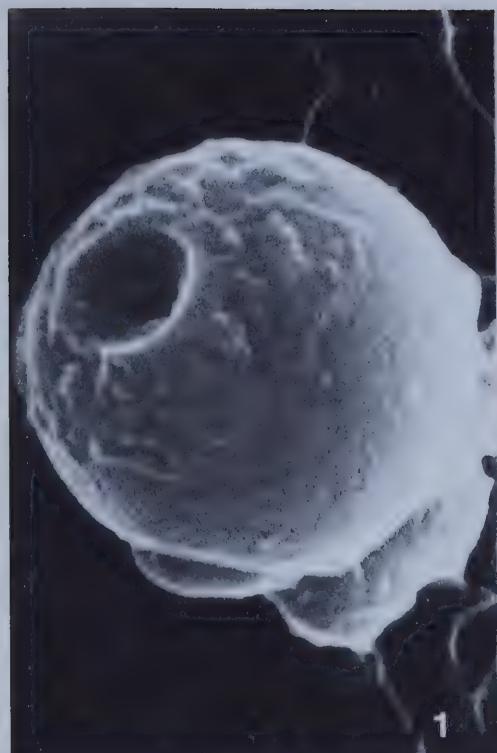








PLATE 2

Figure 4. SEM of the reacted spermatozoon of *Cucumaria lubrica*.

Note: extensive acrosomal process (arrow), X 12,250.

Figure 5. SEM of semi-reacted *C. lubrica* sperm. Note: small bleb

at sperm apex (arrow), X 17,500.







### PLATE 3

Figure 6. SEM of the outer surface of an inseminated *Cucumaria pseudocurata* egg. Note: impressions of detached sperm as well as attached sperm (large arrow) and attached spermatozoon tail (small arrow), X 1,200.

Figure 7. SEM of an attached spermatozoon on the surface of a *C. pseudocurata* egg. Note: impression remaining on egg surface in detached region of the flagellum (arrow); and the hollowed-out reacted acrosomal region, X 12,250.









#### PLATE 4

Figure 8. Scanning electron micrograph of attached spermatozoon on the surface of an inseminated *C.pseudocurata* egg. Note: dissolution of egg investment layer around nuclear and mitochondrial regions of sperm; and reacted acrosomal area, X 13,800.

Figure 9. Same as figure 8. Note: impressions on egg investment layer of detached flagellar regions (arrows); reacted acrosomes; and extensive dissolution of the egg envelope surrounding attached spermatozoa, X 8,750.

Figure 10. Same as figure 8. Note: extensive dissolution of egg envelope; and tubular elevation extending from the center of the acrosomal region posteriorly toward the mitochondrion (arrow), X 16,250.

Figure 11. SEM showing impression of detached sperm on surface of *C. pseudocurata* egg. Note: nuclear (N), mitochondrial (M), striated rootlet groove (arrow) and flagellar (F) regions; and extensive egg surface dissolution surrounding sperm impression, X 17,500.







PLATE 5

Figure 12. SEM of *Leptosynapta clarki* egg. Note: outer particulate-fibrous layer (P) and dense laminate fibrous layer (L), X 200.

Figure 13. Same as figure 12. Note: outer investment layers have been removed by heavy agitation thus exposing the underlying microvilli of the plasma membrane (M), X 450.

Figure 14. SEM of *L. clarki* egg showing outer particulate-fibrous layer (P), dense laminate fibrous layer (L) and microvilli of the plasma membrane (M), X 4,200.



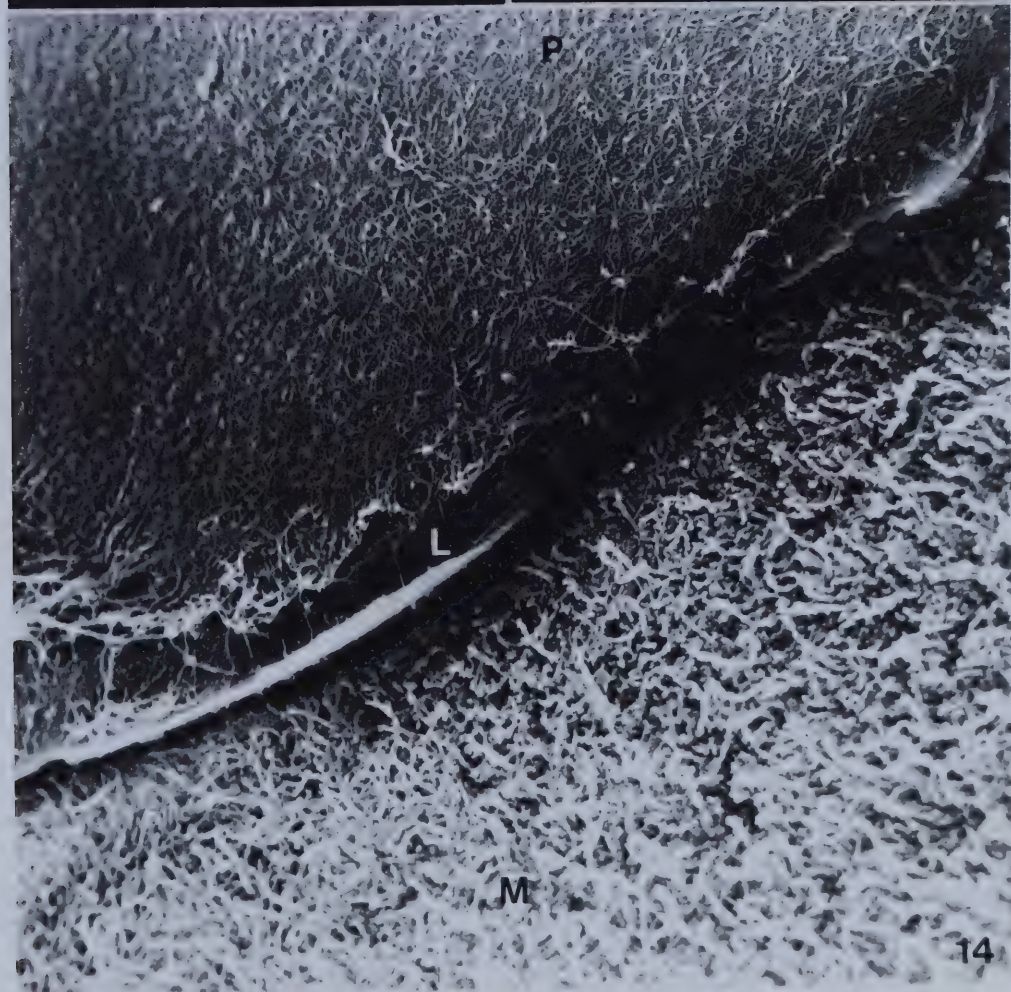
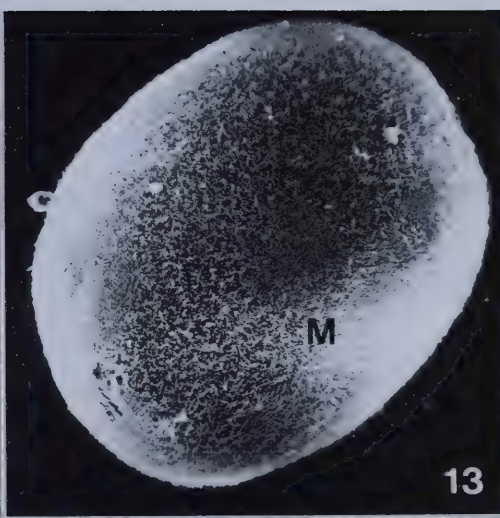
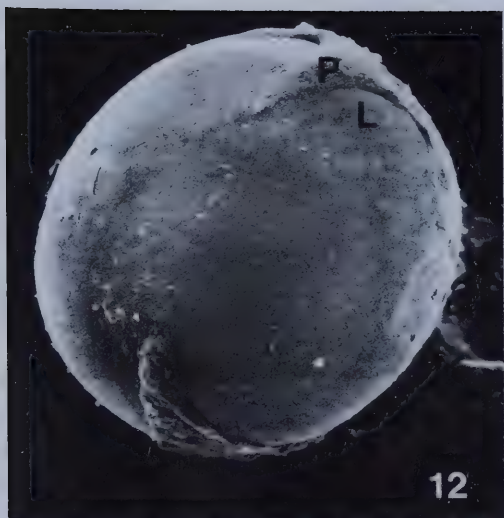






PLATE 6

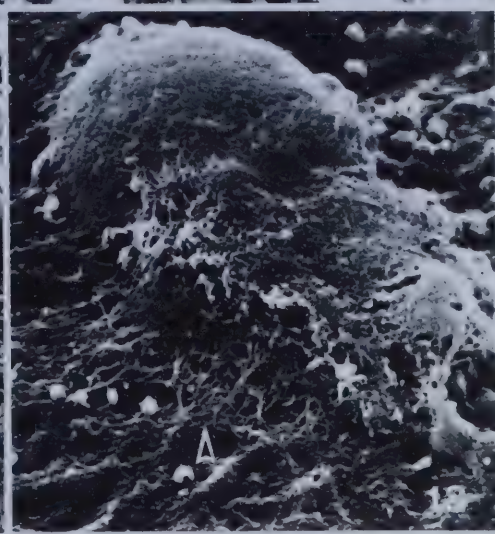
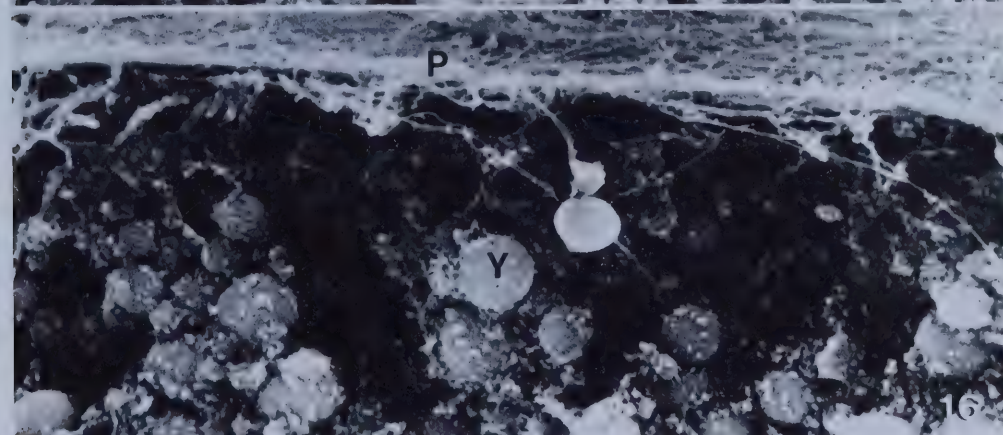
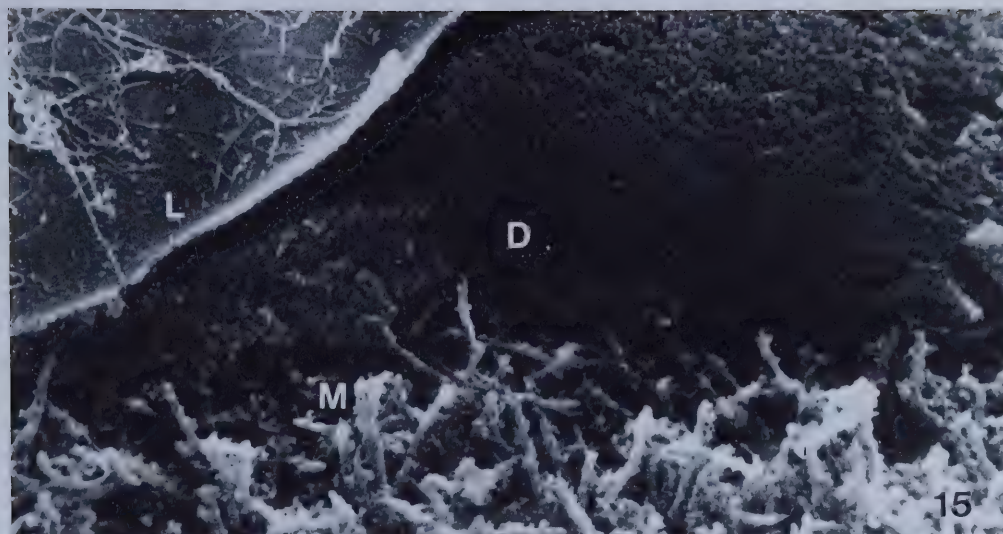
Figure 15. Scanning electron micrograph of *L. clarki* egg showing the dense laminate fibrous layer (L), dense particulate layer (D) and microvilli (M), X 10,500.

Figure 16. Same as figure 15. Note: outer particulate-fibrous layer (P) and yolk granules (Y) of the egg cortical layer, X 9,750.

Figure 17. SEM of follicular cells (arrows) on surface of *L. clarki*, X 3,500.

Figure 18. Same as figure 17. Note: highly branched, thin cytoplasmic processes extending from the follicular cell (arrow), X 10,500.











## PLATE 7

Figure 19. Transmission electron micrograph (TEM) of the investment layers of the *L. clarki* egg. Note: outer particulate-fibrous layer (P), follicular cell process (FC), dense laminate fibrous layer (L), dense particulate layer (D), lucent particulate layer (S), microvilli of the plasmalemma (M) and egg cortical layer (C), X 22,800.

Figure 20. Same as figure 19. Note: desmosome-like structure connecting two follicular cells (arrow); remaining legend is same as for figure 19, X 18,900.

Figure 21. Same as figure 19. Note: separation in the dense laminate fibrous layer (arrows) by space containing electron-dense particles and fibers (area between arrows); and large overlying follicular cell (FC), X 8,050.

Figure 22. TEM of fibers present in the outer particulate-fibrous layer of the *L. clarki* egg, X 66,150.

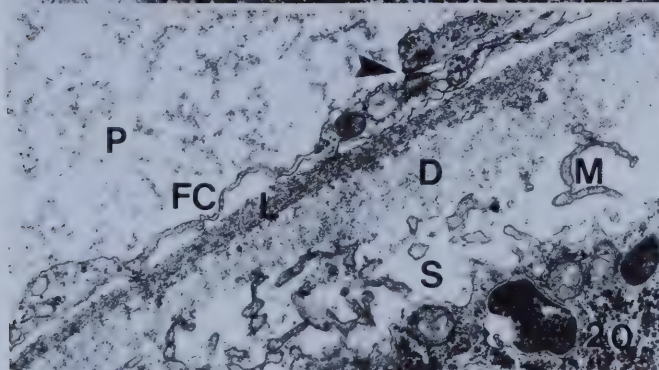
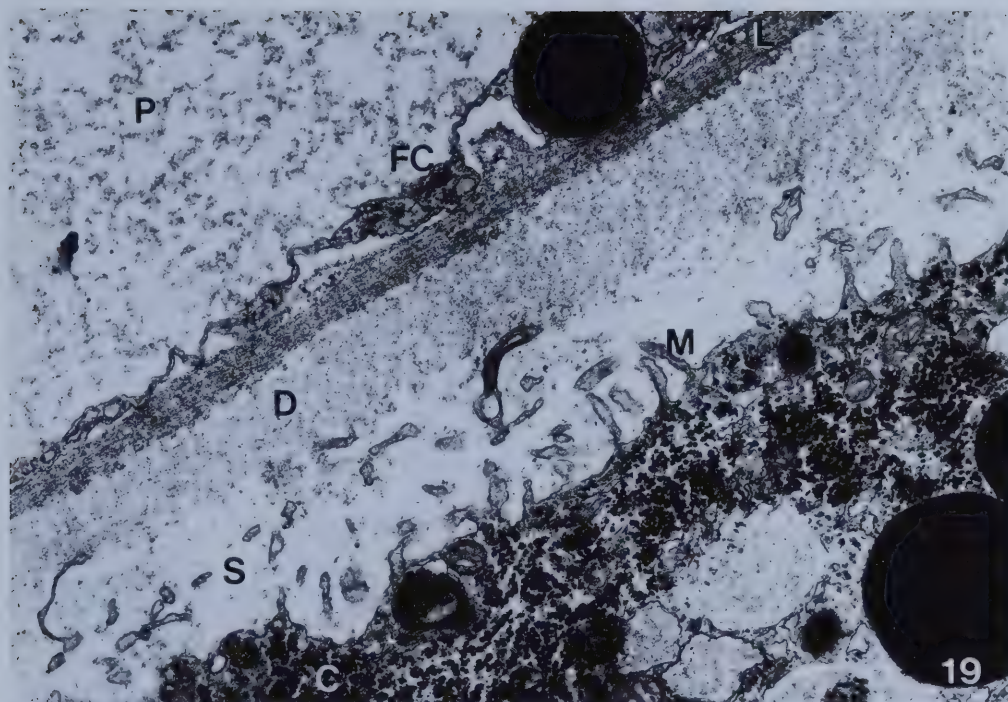






PLATE 8

Figure 23. Scanning electron micrograph of the *Cucumaria lubrica* egg.

Note: dense laminate fibrous layer (L), dense particulate layer (D), microvilli of the plasma membrane (M) and yolk granules (Y) of the egg cortical layer, X 2,650.

Figure 24. Same as figure 23. Note: dense laminate fibrous layer

(L), dense particulate layer (D) and microvilli (M), X 12,250.



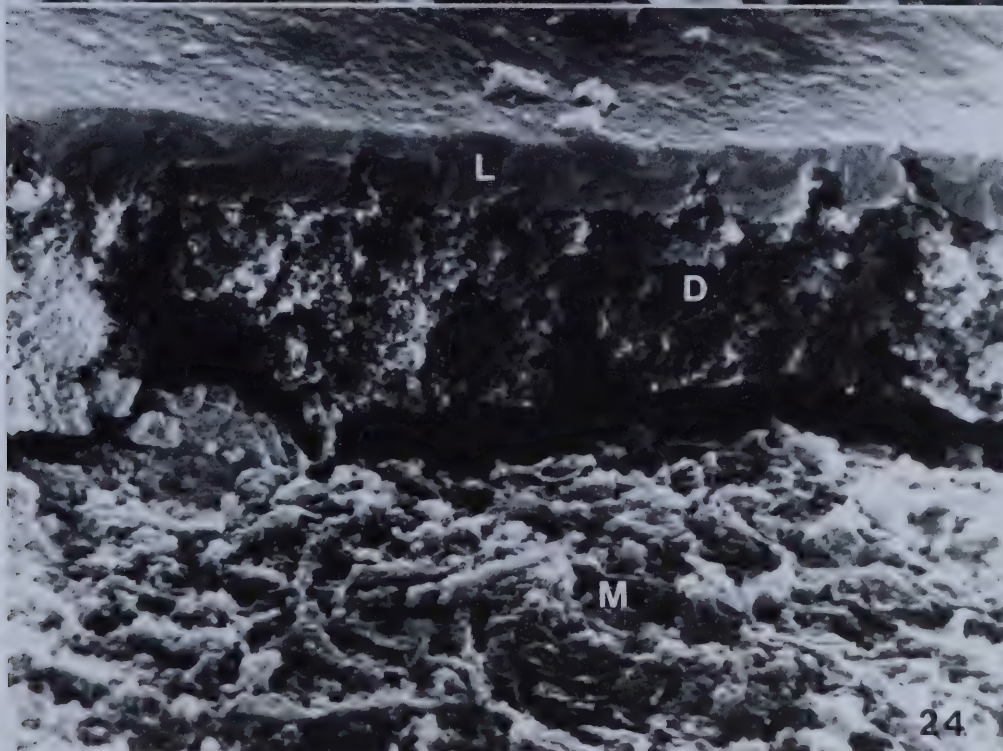
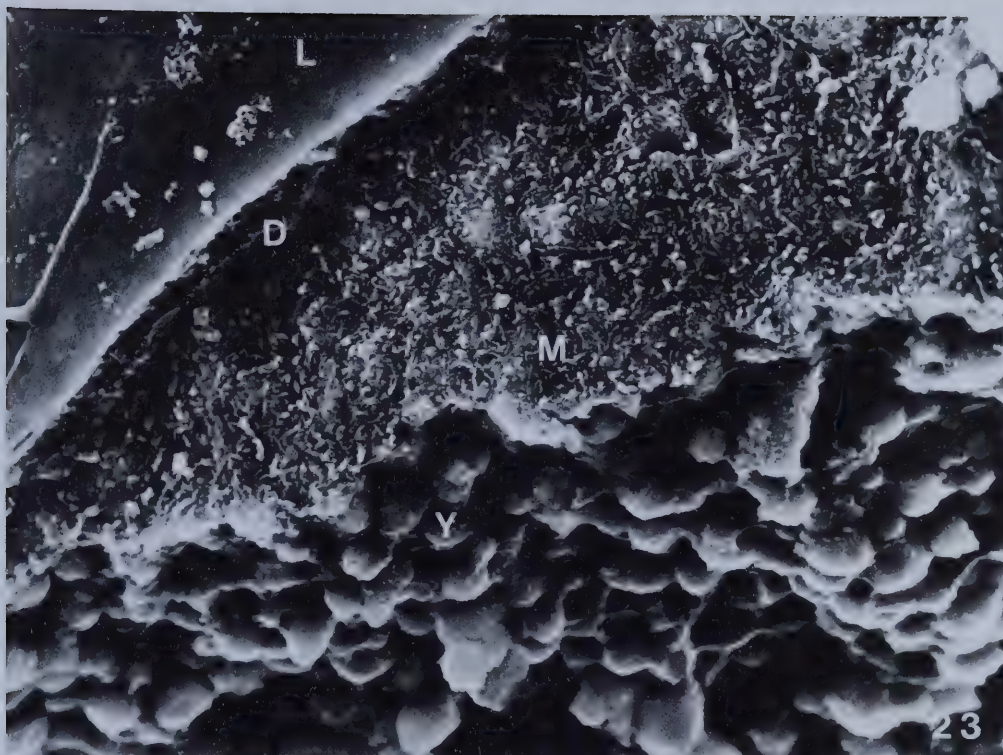








PLATE 9

Figure 25. TEM of investment layers of the *Cucumaria lubrica* egg.

Note: follicular cell (FC), underlying dense laminate fibrous layer (L), dense particulate layer (D) and yolk granules (Y),  
X 9,100.

Figure 26. Same as figure 25. Note: microvilli of the plasma membrane (M); remaining legend is same as for figure 25, X 9,000.

Figure 27. TEM of *C. lubrica* egg showing plasma membrane with microvilli (M) and cortical layer with yolk granules (Y). Note the lack of cortical granules, X 24,300.

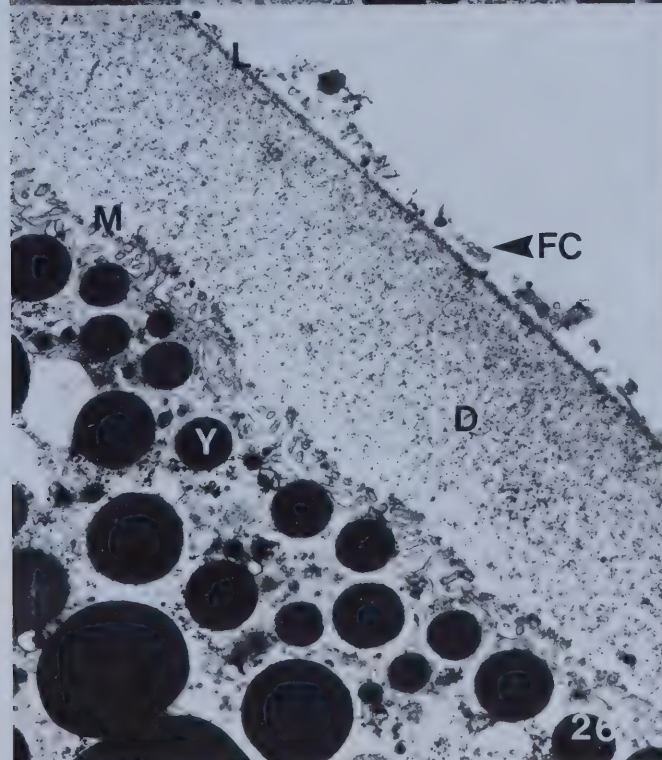
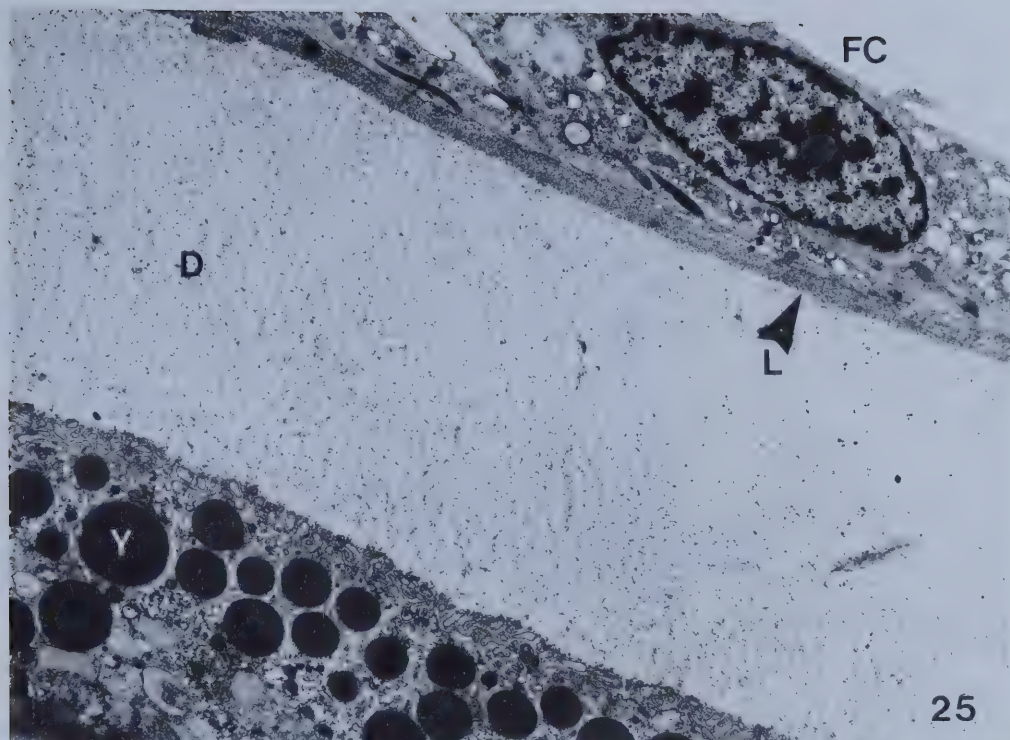






PLATE 10

Figure 28. Scanning electron micrograph of the outer investment layer of the *Cucumaria pseudocurata* egg within an ovarian tubule. Note: tall columnar epithelial cells of the ovary (O), dense laminate layer of the oocyte (L), small electron-dense granules in egg cortical layer (X) and large yolk granules also in the egg cortical layer (Y), X 4,200.

Figure 29. SEM of the outer layers of the *C. pseudocurata* egg. Note: dense laminate layer (L), basal zone of the dense laminate layer (arrows), vesicles of the egg cortical layer (V), small electron-dense granules (X) and yolk granules (Y) in the egg cortical layer, X 4,200.











## PLATE 11

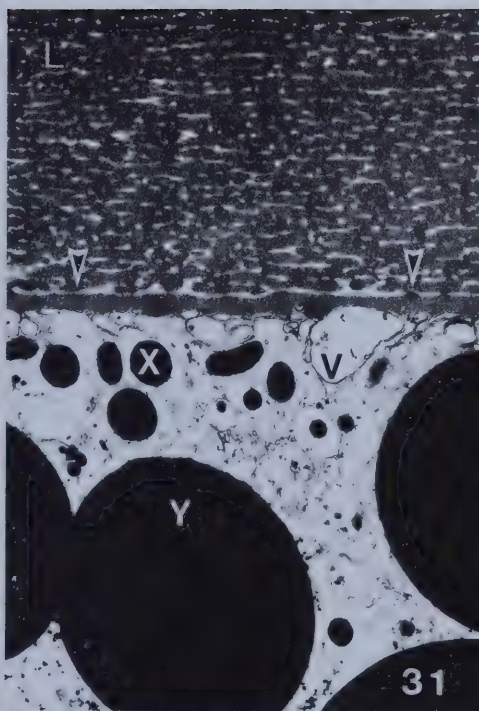
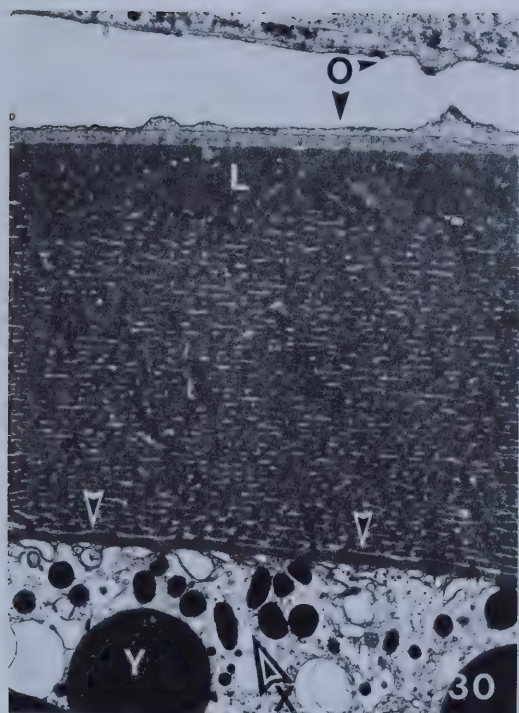
Figure 30. Transmission electron micrograph of the outer layers of the *C. pseudocurata* egg within an ovarian tubule. Note: ovarian tissue (O), dense laminate layer of the egg (L), basal zone of the dense laminate layer (arrows), small dense granules in egg cortical layer (X) and yolk granules (Y), X 7,700.

Figure 31. TEM of the outer layers of the *C. pseudocurata* egg.

Note: vesicles beneath the plasma membrane (V); remaining legend is same as for figure 30, X 10,800.

Figure 32. SEM of the surface of the inseminated *C. pseudocurata* egg.

Note: impression of detached sperm (S), dense laminate layer of the egg (L), fibrous layer (F) which forms between laminate layer and cell membrane, small dense granules of egg cortical layer (X) and yolk granules (Y), X 4,550.





## Chapter VIII

### GENERAL DISCUSSION AND CONCLUSION





## GENERAL DISCUSSION AND CONCLUSION

This chapter is an attempt to draw together the discussions from the preceding six chapters in a manner so as to answer the questions set forth in the general introduction. Summaries, conclusions and hypotheses will be presented in the same order as the questions.

1) How is spermatogenesis in the class Holothuroidea comparable to that reported in other echinoderm classes; and is there variation in the spermatogenic process within the holothurian class?

Except in the case of the echinoids *Arbacia punctulata* and *Strongylocentrotus purpuratus* (Longo and Anderson, 1969) there is no detailed comparative work available on the phylum. Nevertheless, general observations are available in the class Asteroidea. In the asteroid *Leptasterias* developing germinal cells form fingers or colonnettes (Cognetti and Delavault, 1962) which bulge out from the germinal epithelium into the lumen of the testes (Smith, 1971). The cells of the colonnettes are layered according to maturity and proceed from spermatogonia at the base to mature spermatozoa at the luminal tip. In the echinoids (Longo and Anderson, 1969) and holothurians (present study) the germinal cells are arranged in a series of cell stages progressing from spermatogonia (normally in contact with the testicular wall) to spermatocytes (located among and lumenally to the spermatogonia) to spermatids and spermatozoa (occurring centrally in the testicular lumen). Developing cells are not separated into definite zones but are generally more mature as they proceed from the gonadal wall toward the lumen.



### *Morphogenesis of Acrosomal Region*

Information concerning acrosomal granule morphogenesis in the echinoderms is very sparse. It is generally agreed that the Golgi complex is associated with the formation of proacrosomal vesicles. These vesicles probably coalesce to form the initial acrosomal granule during the late spermatocyte-early spermatid stage of spermatogenesis. The initial granule in the asteroid *Asterina* contains a central region of sparse, randomly oriented, dense material surrounded by a region of material in a radial micellar arrangement. The centrally located material condenses as spermiogenesis proceeds and becomes more electron-dense than the peripheral region (Dan and Sirakami, 1971). In the echinoids *Arbacia* and *Strongylocentrotus* the initial granule appears as a membrane-bound vesicle void of electron-dense material. The granule, at a late stage of spermiogenesis, consists of a fine granular homogeneous matrix which contains no characteristic substructure (Longo and Anderson, 1969). In the holothurian *Cucumaria lubrica*, acrosomal formation is similar to that reported in *Asterina*. The initial granule, which is irregularly circular and enclosed by a membrane, consists of a dense homogeneous material with a reticular consistency. The granule material loses the reticular appearance during migration toward the apical sperm surface and becomes less electron-dense. By the time the granule reaches the nuclear region, destined to become the acrosomal depression, it consists of a homogeneous material except for an electron-dense sphere displaced anteriorly.

In the present investigation, it was found that morphogenesis of the acrosomal granule in *Leptosynapta clarki* differs in details from



other echinoderm species. The initial granule is irregularly circular, surrounded by a limiting membrane and consists of a heterogeneous material distinctly segregated into a dense reticular peripheral zone and a less dense vesicular central zone. As the granule matures, the central vesicular structures break down, forming a zone composed of a homogeneous material less electron-dense than that of the peripheral zone, which has become less reticular in nature. Later in morphogenesis the granule becomes relatively circular in shape and develops four or five concentric dense bands in the central zone. Two electron-dense, cup-shaped bands occur in the posterior region of the granules of both *L. clarki* and *C. lubrica*. These bands, which can be equated to similar structures within the asteroid granule, have been shown by Dan and Hagiwara (1967) to be involved in the acrosomal reaction.

Morphogenesis of the acrosomal granule in *Cucumaria pseudocurata* is likewise unique to previously reported echinoderms. The granule begins as a large irregularly circular structure containing a highly sparse, fine reticular material. In the late spermatocyte the granule contents have condensed from a sparse reticulate state to a more electron-dense particulate-fibrous nature. As in *L. clarki*, the granule membrane destined to become the adnuclear surface becomes overlaid with a thin layer of dense materials. As the granule matures, this dense material becomes less osmiophilic and localized to the inside of the adnuclear granule membrane. The granule changes from irregularly circular in shape to a structure with its anterior-posterior surfaces flared out into the surrounding periacrosomal material. The osmiophilic material of the adnuclear surface progresses to the state of an incomplete membrane-like



structure which extends from the anterior-posterior inner surfaces around the dorsal face of the granule. This structure could be correlated to the cup-shaped bands in the posterior granule regions of *L. clarki* and *C. lubrica*.

Encircling the acrosomal granule in *L. clarki*, *C. lubrica* and *C. pseudocurata*, within a nuclear depression, is a periacrosomal layer generally composed of a reticular material. This material appears to arise from the cytoplasm lying in the region of the nuclear depression rather than from other areas of the cell.

It is generally believed in echinoderms that the acrosomal granule is formed in the basal cytoplasm region of the spermatocyte-spermatid stage and migrates to the apical end of the cell by time of maturity. In the asteroid *Asterina*, the migrating granule maintains no specific orientation with respect to the nucleus. However, the surface on which dense materials are deposited is always at the side farther from the Golgi complex (Dan and Sirakami, 1971). In *L. clarki* the dense surface of the granule appears to remain in close association with the nuclear envelope throughout development. In *C. pseudocurata* the dense surface of the granule faces away from the nuclear envelope during migration and rotates within the nuclear depression into an adnuclear position at the spermatid stage. Acrosomal rotation of this type has not been previously reported in echinoderm species.

#### *Morphogenesis of Mitochondrial Region*

In all three holothurian species investigated in the present study, mitochondrial elements transform from small ovoid forms in spermatogonia





and spermatocytes to a single large structure in spermatids. Two hypotheses are available to explain this final shape of the mitochondrion. The first possibility is that mitochondrial elements observed in spermatogonia and spermatocytes fuse to form one single organelle, as reported in echinoids (Longo and Anderson, 1969). A second possibility is that only one mitochondrion exists in the cell throughout the entire process of spermatogenesis. The mitochondrion, in the early stages, would be highly branched, consisting of many greatly folded tubular units. This model could explain the many small ovoid mitochondria observed in sections of earlier stages of spermatogenesis. It is then conceivable that as the germinal cell matures, the mitochondrion condenses into a single compact organelle lying at the base of the nucleus. Preliminary data obtained from serial sections of early spermatids of *L. clarki* and *C. pseudocurata* indicate that this model is feasible. Hoffmann and Avers (1973) have shown that a single tubular mitochondrion rather than numerous separate units exists in the yeast cell and have indicated that a similar situation is noted in mammalian cells.

#### *Morphogenesis of Nuclear Region*

During the process of spermatogenesis, in all three holothurian species, the nucleus becomes relatively circular. In *L. clarki* the circular shape is maintained throughout spermiogenesis and remains unchanged in the spermatozoon. Spermatids of *C. lubrica* and *C. pseudocurata*, on the other hand, undergo an elongation process which results in a torpedo-shaped (cylindrical) nucleus in the *C. lubrica* sperm and a tabloid-shaped nucleus in *C. pseudocurata*. A similar nuclear elongation



process is observed in the echinoids *Arbacia* and *Strongylocentrotus* (Longo and Anderson, 1969). Nuclear elongation in the holothurians and echinoids does not appear to be accompanied by the presence of microtubules but is probably due to internal condensation of the chromatin material of the nucleus.

In *L. clarki* and *C. lubrica* spermatids, nuclear indentations form at the posterior (centriolar fossa) and anterior (acrosomal depression) regions. Extending from the nuclear surface of the proximal centriole into the posterior centriolar fossa is a dense projection with a fibrous consistency in *L. clarki* and a tubular-fibrous consistency in *C. lubrica*. The formation of the fibrous projections parallel that of the centriolar fossa in the early spermatid. The projections gradually become less prominent during late spermiogenesis but remain in a reduced form in the mature spermatozoon. Longo and Anderson (1969) reported that similar projections occurred in echinoid spermatids but were absent from spermatozoa. It was ventured that they were instrumental in the formation of the centriolar fossa. A dense fibrous structure extending from the proximal centriole and similar to the fibrous arms in *L. clarki*, *C. lubrica* and echinoids, develops in the early spermatocyte stage of *C. pseudocurata*. In this holothurian species the fibrous arm is a transitory structure which is instrumental in the formation of the extensive striated rootlet-like structure observed in the spermatozoon. It is possible that the fibrous arms of *L. clarki* and *C. lubrica* are likewise involved in the morphogenesis of the transitory striated rootlet-like structures noted in the centriolar region of the spermatocytes of these two species.



### *Tubular and Chromatoid Bodies*

Two cytoplasmic organelles not previously reported in echinoderms have now been observed in germinal cells of holothurians. The spermatogonia of *C. lubrica* contain a tubular body which consists of parallel microtubules. This structure has been termed the Crystalloid of Lubarsch (Lubarsch, 1896) and has been observed in human (Nagano, 1969; Rowley et al., 1971) as well as rooster (Nagano, 1969) spermatogonia. The ultimate fate and function of this structure remains obscure. Tubular bodies were never found in *L. clarki* or *C. pseudocurata* germinal cells. In *L. clarki* spermatocytes and *C. pseudocurata* spermatids there occurs a densely staining chromatoid body. The structure is of a honeycomb configuration with a fine granular consistency. The body is frequently surrounded by mitochondria and normally lies closely associated with the nuclear envelope. Similar chromatoid bodies have been reported in various vertebrate and invertebrate germinal cells (Sud, 1961a,b; Fawcett et al., 1970; Comings and Okada, 1972; Fawcett, 1972). Previous instances in invertebrates (Fawcett, 1972) have been limited to the classes Arachnida, Crustacea and Insecta of the phylum Arthropoda. Fawcett (1972) indicates that the chromatoid body is primarily a basic protein which may contain small amounts of RNA at certain times and that there is some experimental evidence that it is essential for germinal cell formation. The initial speculation was that the body functioned as precursor material for the formation of tail components but since its discovery in vertebrate oocytes this theory has been discredited. Its function is presently unknown (Fawcett, 1972).





Fawcett (1972) has suggested that the chromatoid body originates, at least in mammals, as loosely packed dense materials within mitochondrial clusters of spermatocytes. The mitochondria gradually disperse as the dense materials of the chromatoid body aggregate into a single mass. As the cell matures into a spermatid the body forms a close association with the nuclear envelope and migrates round to the base of the flagellum. At this position the body forms a ring around the flagellum adjacent to the annulus and finally disperses leaving no structural remnants in the mature spermatozoon. The fact that the chromatoid body appears to have a parallel in the process of oogenesis (Eddy and Ito, 1971; Mahowald, 1968, 1971) adds to the curiosity concerning this organelle.

Many of the cellular changes observed during spermiogenesis in *C. pseudocurata* are unparalleled in the class as well as in the phylum. Morphogenesis of the extensive striated rootlet and the alteration of cell shape in late spermiogenesis is very similar to that reported during sperm formation in mammalian species. In the spermatocyte stage several structures develop which are presumably for anchorage and/or stability during the developmental stages of the flagellum. These structures either disappear in later stages (basal foot, dense fibers radiating from basal foot), transform into mature structures (dense arm of proximal centriole, filamentous structures extending from distal to proximal centriole) or persist throughout spermiogenesis (satellite materials of distal centriole).

Basically, therefore, spermatogenesis in holothurians is comparable in several aspects with other echinoderm species, with many differences also being evident. As shown from this research on only three species,



great variation also occurs within the class Holothuroidea.

2) Do holothurian spermatozoa conform to the primitive sperm type exhibited by other echinoderm species; and if not, then at which stages during spermatogenesis are modifications introduced and how are these alterations reflected in the spermatozoa?

Franzén (1967a,b, 1970) has noted a definite relation between sperm morphology, spermiogenesis and the biology of propagation. Spermatozoa which are produced by species that retain the primitive mode of sperm discharge directly into the water and fertilize externally have been termed the primitive type. This type of sperm is generally a small cell with a short rounded or conical nucleus, a midpiece consisting from one to a few mitochondria and a long flagellum. The apical end of the sperm houses the acrosome which has the capabilities of undergoing an acrosomal reaction as a consequence of contact with the egg. The tip of the spermatozoon will be the first region of the cell to contact the oocyte at the time of fertilization. "If the spermatozoa are not discharged freely into the water for the fertilization of the eggs but are transferred directly to the receiving female animal by spermatophores or by organs of copulation, the morphology of the spermatozoon is altered in some way or other" (Franzén, 1970). In those species which have sperm with an altered morphology it is possible to follow the earliest and most conspicuous steps in the spermatozoon's modification by carefully studying the process of spermiogenesis.

Morphologically, the spermatozoon of *L. clarki* is of the primitive type and the events noted during spermatogenesis are typical of the process in those organisms that produce primitive sperm. The sperm of



*C. lubrica* deviates from the primitive type in that the nucleus is elongate and the mitochondrial elements are rearranged along the posterior portion of the nucleus rather than positioned between the nucleus and flagellum. Nuclear elongation and mitochondrial rearrangement first becomes evident in the intermediate spermatid stage. A tapering of the posterior  $1.6\ \mu$  of the nucleus accompanies the re-positioning of the mitochondrion.

The spermatozoon of *C. pseudocurata* deviates from the structural pattern observed in the primitive sperm in that it is elongated and dorsal-ventrally compressed, contains an extensive striated rootlet-like structure in the midpiece, has an enlargement and rearrangement of mitochondrial elements of the midpiece, and contains an additional fiber in the flagellum. The stage where it becomes evident that the spermatogenic process of *C. pseudocurata* is deviating from the characteristic pattern observed in formation of the primitive sperm, is in the early spermatocyte. At this point the formative materials for the striated rootlet are being deposited in the regions of the proximal and distal centrioles. Basic cell shape first becomes altered in the intermediate spermatid stage where there is a corresponding compression and elongation of the nucleus. Compression occurs in the dorso-ventral plane, whereas elongation is along the anterior-posterior axis. In the late spermatid there is a gradual shift of remaining cytoplasm to the posterior-dorsal region of the cell. This shift in cytoplasm is accompanied by an increase in nuclear compression and elongation. The dorsal groove which houses the striated rootlet originates on the dorsomedial surface of the nucleus in the intermediate spermatid and elongates posteriorly as the cell



matures. Through this cytoplasmic shifting process the striated rootlet comes to lie parallel with and tightly situated within the groove. In the late spermatid stage the small ovoid mitochondria transform into a single large mitochondrion. Mitochondrial elements surround the centrioles and the posterior region of the striated rootlet as well as extending through the dorsal rootlet groove on the dorsal sperm surface. Mitochondrial elements extend for approximately two-thirds of the length of the groove and terminate posterior to the acrosomal region.

An interesting structure develops during the spermatogenic process in *C. pseudocurata* and completely degenerates by the time the cell reaches maturity. The structure is a basal foot-like projection which extends laterally from the wall of the distal centriole. Formation of the basal foot first becomes evident in the early spermatocyte stage. In the late spermatocyte the basal foot reaches maturity and consists of a dense cap, separated from a stalk by a less osmiophilic space. The stalk is separated by another less osmiophilic space from the two dense connecting regions attached to the centriolar wall. Fibers radiate from the cap region of the foot and exist in a tight zone between the foot and the proximal centriole. In the early spermatid stage the basal foot and radiating fibers have totally degenerated. It has been postulated in other cells that the basal foot is a possible mechanosensitive structure instrumental in the control of directional movement of cilia (Thurm, 1968; Flock, 1971). Ciliated cells which contain basal feet show a fixed directional ciliary beat which is related to the orientation of the feet. The questions that arise are why should a developing sperm cell be equipped with such a structure and for what reason does it





degenerate? There are at least three possibilities: 1) the basal foot is strictly an anchorage/stability structure only necessary during the formative stages of the flagellum and striated rootlet, 2) the foot is strictly a mechanosensitive device important in the initial movements of the flagellum, or 3) the foot exhibits a combination of the above two functions.

3) Does spermatozoan morphology differ significantly within the holothurians; and if so, is it possible to correlate changes in spermatozoan morphology with an altered biology of propagation or an alternative factor such as egg investments?

#### *Comparative Aspects of Holothurian Spermatozoan Structure*

Basic sperm morphology (based on light and scanning electron microscopy) has been described for at least 20 species of holothurians: *Anapta gracilis*, *Chimotota incongna*, *Cucumaria cucumis*, *Cucumaria elongata*, *Cucumaria frondosa*, *Cucumaria miniata*, *Cucumaria piperata*, *Cucumaria planci*, *Eupentacta pseudoquinquesemita*, *Holothuria atra*, *Holothuria poli*, *Holothuria tubulosa*, *Holothururua edulis*, *Mesothuria intestinalis*, *Molpadia oolitica*, *Parastichopus californicus*, *Psolus chitonoides*, *Synapta inhaerens*, *Thyone briareus*, *Trochostoma thompson*. The sperm of these species are reviewed by Chia et al. (in press).

All of these spermatozoa, without exception, contain a relatively small spherical-shaped head measuring 2 to 4  $\mu$  in diameter (Chia et al., in press) positioned anteriorly to a small midpiece connected to a relatively long flagellum. The present study has demonstrated that two additional sperm shapes, cylindrical (*Cucumaria lubrica*) and tabloid



(*Cucumaria pseudocurata*), exist in the class.

The significant structural differences among the three spermatozoa examined in the present study occur in the shape of the head or nuclear region, substructure and location of the acrosome, components of the midpiece and number of tubules in the flagellum.

*Nuclear region.* The nucleus of the *L. clarki* sperm is irregularly circular in shape and measures approximately  $2.9\ \mu$  in width. The anterior-posterior axis is shorter due to the anterior acrosomal depression and posterior centriolar fossa. The *C. lubrica* nucleus is elongated measuring about  $6.8\ \mu$  in length and  $1.4\ \mu$  at its greatest diameter. The posterior  $1.6\ \mu$  of the nucleus is tapered to a diameter of  $0.5\ \mu$ . The nucleus is indented anteriorly by an acrosomal depression and posteriorly by a centriolar fossa. In *C. pseudocurata* the nucleus is dorso-ventrally compressed measuring approximately  $5.5\ \mu$  in length,  $1.2\ \mu$  in width and  $0.8\ \mu$  in depth. An extensive acrosomal depression is noted on the ventral surface. The indentation is medially located along the anterior-posterior axis of the spermatozoon and extends almost completely through the depth of the nucleus. The nucleus is indented posteriorly along the dorsal surface by the striated rootlet groove. The nucleus is tapered at the anterior and posterior ends and reaches its maximum dimensions slightly posterior to the acrosomal depression.

*Acrosomal region.* In *L. clarki* the acrosomal granule is relatively circular measuring  $0.7\ \mu$  in diameter and is housed in a nuclear acrosomal depression. The granule is membrane-bound and consists of electron-dense material which in the central region is arranged in concentric lamellae. Five such very osmiophilic areas alternate with areas of much less



electron-dense material. Two osmiophilic bands occasionally occur in the posterior region of the granule. The acrosomal granule in *C. lubrica* measures about  $0.6\ \mu$  in width and  $0.5\ \mu$  in length and in the majority of sections is membrane-bound. The limiting membrane is frequently thrown into a number of small folds at the apex of the granule, giving a serrated effect to the anterior end of the acrosome. Granule contents are of a homogeneous nature except for a dense osmiophilic sphere displaced toward the anterior surface. As in *L. clarki*, two osmiophilic, cup-shaped bands occur in the posterior region of the granule and have been termed primary membrane precursors which are involved in the asteroid acrosomal reaction (Dan and Hagiwara, 1967).

In *C. pseudocurata* the acrosomal granule is composed of a dense homogeneous particulate material surrounded by a limiting membrane. The granule is irregular in shape with the width (anterior-posterior axis) measuring  $730\ \text{m}\mu$  and the depth (dorsal-ventral axis) being  $425\ \text{m}\mu$ . The anterior-posterior surfaces flare out forming pockets in the surrounding periacrosomal material. The external (ventral) granule surface bulges out to form a close association with the cell membrane and the internal (dorsal) surface is indented forming an inward depression. Ventral to this depression, within the granule, is a small area containing particulate-fibrous material of a greater osmiophilic density than the surrounding material. To the inside of the granule limiting membrane there is an incomplete membrane-like structure which extends from the anterior-posterior surfaces around the dorsal face. This structure can possibly be equated to the cup-shaped bands noted in the posterior granule region in *L. clarki* and *C. lubrica*.





Completely surrounding the acrosomal granule in the sperm of all three species is a periacrosomal layer of basically homogeneous reticular material less electron-dense than the granule. In *L. clarki* an ill-defined area containing a small amount of fibrous material is located posterior to the granule within the periacrosomal layer. This area forms no obvious subdepression and is infrequently observed. A similar fibrous area is noted in *C. lubrica*. In *C. pseudocurata*, areas of the periacrosomal layer ventral to the pockets (sandwiched between the nuclear envelope and the granule membrane) are more dense and granular than the remaining periacrosomal material. These areas could be correlated with the material composing the fibrous plate precursor of asteroid acrosomes (Dan and Hagiwara, 1967). Dorsomedial to the granule is a distinct particulate-fibrous region lodged within the granule depression. This region corresponds to the periacrosomal fibrous zones noted in *L. clarki* and *C. lubrica* and is presumed to be the precursor of the acrosomal filament.

*Midpiece region.* The midpiece regions of the *L. clarki* and *C. lubrica* spermatozoa are very similar except for the arrangement of the mitochondrial elements. The major difference is that in *L. clarki* the entire nucleus lies anterior to the mitochondrion whereas, in *C. lubrica*, the tapered posterior 1.6  $\mu$  of the nucleus is surrounded by the mitochondrion. Both species contain typical proximal and distal centrioles oriented perpendicular to one another lodged within the mitochondrial mass. A dense fibrous arm projects from the proximal centriole into the centriolar fossa in both species. The distal centriole, which is connected to a series of satellite projections and y-shaped membrane



doublet connectives, gives rise to a typical 9+2 flagellum. The satellite of the distal centriole consists of nine radiating fibers each of which branches into two secondary fibers and in turn branch into fine tertiary fibers which form a network with adjacent tertiary fibers. This network, with which microtubules are closely associated, is very extensive and lies in close proximity to mitochondrial elements.

The midpiece of the *C. pseudocurata* spermatozoon structurally is more complex than that of the other two species investigated. The mitochondrial mass lies immediately posterior to the nucleus and surrounds the proximal and distal centrioles, satellite projections and much of the striated rootlet. The greatest mitochondrial mass occurs on the dorsal side of the sperm where it lies within the rootlet groove and extends for about two-thirds of its length. The centrioles are situated posterior to the nucleus, perpendicular to one another with the distal centriole giving rise to a flagellum. The distal centriole contains the typical nine rows of three tubules, whereas the proximal centriole appears to consist of nine rows of five tubules. Extending anteriorly from the distal centriole to just below the acrosomal region is a striated rootlet. The rootlet originates at the proximal surface of the distal centriole and encompasses the proximal centriole as it projects anteriorly toward the sperm apex. The structure and location of the rootlet suggests that it may function as an anchoring and/or stabilizing device for the distal centriole and flagellum. In *C. pseudocurata* both the proximal and distal centrioles contain satellite projections. The satellite materials of the proximal centriole assume various shapes ranging from slender extensions protruding from the triplets to large dumbbell-



shaped spheres connected to the triplets by thin bridges. The satellite material of the distal centriole, which is a continuation from the proximal centriole, is arranged in more of an ordered manner and is similar in configuration to that observed in *L. clarki* and *C. lubrica*.

*Flagellar region.* In *L. clarki* and *C. lubrica* the tail flagellum contains the typical 9+2 microtubule arrangement. In *C. pseudocurata* a third central tubule exists in the midtail region between and slightly peripheral to the central pair, thus giving a 9+3 pattern to the flagellum. This third tubule is normally in direct contact with the central pair but is separated by a small space from one or the other in many sections. The 9+3 pattern was never observed in the basal or tip regions of the tail, indicating that the third tubule originates and terminates along the midsection of the tail.

Great variation is noted throughout the animal kingdom in the microtubule pattern of the motile sperm flagellum. The arrangement is a 9+9+0 in mayflies, 9+0 in *Vejevís* and *Hadurus* (scorpions), 9+9+2 in the majority of insects and mammals, 9+9+1 in mosquito species, 9+1 in many flatworms and *Centrorides* (scorpion), and 9+7 in two species of caddis flies (Phillips, 1970, 1974). A 9+3 arrangement has been observed in a fungus gnat and several species of spiders (Phillips, 1970, 1974). In *Pisaurina* (spider) the three central tubules originate at the center base of the distal centriole and run throughout the length of the tail (Reger, 1970). The 9+3 pattern of *C. pseudocurata* differs from the above species in that the additional central tubule originates and terminates in the midsection of the flagellum.



### *Correlation of Sperm Structure with Biology of Propagation*

Franzén (1967a, 1970) and Afzelius (1972) have discussed in length the relationships that exist between spermatozoan morphology and the biology of fertilization. It has been shown that the majority of marine invertebrate species investigated conform to the hypothesis that modified sperm structure can be correlated to a modification in the process of propagation; namely, sperm transport from the male to the egg of the female (packaging of sperm and movement of sperm through the female reproductive tract). An alternative factor that may be considered as a feasible cause for sperm modification is egg investments. It is logical that species with eggs containing complex and specialized investment layers would likewise require a modified spermatozoon for penetration and ultimately fertilization.

Four main factors, therefore, can be considered as possible causes for structural modifications in mature sperm: 1) packaging of sperm for release from the male and transport to the egg (Afzelius, 1972), 2) movement of sperm through media of varied viscosities (Afzelius, 1972), 3) movement of sperm through the female reproductive tract (Afzelius, 1972), and 4) adaptation to a particular egg envelope (present study).

*Leptosynapta clarki*. *L. clarki* is an internal (ovarian brooder with internal fertilization, which produces a sperm typical of the primitive type. During the spawning season, small groups of individuals lie in close contact with their anterior ends lying in cylindrical depressions in the sand substratum. These sand pockets contain sea water and could act as closed reservoirs in which spermatozoa could move from males to females (Everingham, 1961). The manner of sperm entry into the female





is unknown. Everingham (1961) suggests that the sperm might enter the ovarian lumen through the gonopore and fertilize the eggs or they might enter into the coelomic cavity through the rectum or small perforations in the body wall.

According to Afzelius (1972) several other organisms exhibit internal fertilization and produce a primitive type sperm: *Tealia felina* (sea anemone), *Aleyonium* (coral), *Unio*, *Anodonta*, *Sphaerium*, *Teredo* (mussels) and *Polycarpa* (tunicate). Afzelius proposes that in these species, the internal fertilization habit is not extensively different from that of external fertilization, in that large numbers of sperm are produced and released into the water, which are then drawn into the female by an inhalant current. Water currents are constantly circulating through these animals, presenting an open system directly connected with the external sea water environment. In *L. clarki* the internal fertilization habit is different from that of external fertilization in that water currents are not constantly circulated, thus presenting a closed system with respect to the surrounding sea water. Also, in *L. clarki*, the spermatozoa must move through the female reproductive system prior to fertilization, whether sperm entry is through the gonopore or rectum.

*L. clarki*, therefore, is a typical internal fertilizer with a female tract that sperm must travel through prior to fertilization, which produces a primitive type spermatozoon typical of an external fertilizier. Thus, Franzén's hypothesis on sperm structure is not adequate to explain sperm structure in this particular species.

*Cucumaria lubrica*. *C. lubrica* is an external ventral surface brooder with external fertilization which produces a sperm that deviates



from the primitive type in that the nucleus is elongate and the mitochondrial elements are rearranged around the posterior region of the nucleus. This species occurs in swift water currents in great abundance (5000/m<sup>2</sup>) on subtidal rocks. It has been noted in the laboratory that following spawning, the sperm remain together in bundles for an extended length of time before they are suspended. The elongated sperm head may reflect a specific adaptation for the convenience of packaging of sperm bundles. Spermatophore-like structures containing elongate spermatozoa are formed in many organisms: *Littorina* (Prosobranchia) (Buckland-Nicks, 1973), *Hadrumus* (Scorpion) (Jespersen and Hartwick, 1973), *Octopus* (Cephalopod) (Franzén, 1967b; Longo and Anderson, 1970), and *Sibolinum* (Pogonophora) (Franzén, 1973).

Many organisms (such as mammals and gastropods) with internal fertilization produce modified sperm in which the mitochondrial elements extend down the sides of the flagellum. In *C. lubrica* as well as in some turbellarians (Silveira and Porter, 1964), ostracods (Reger and Florendo, 1969) and Nemertines (Afzelius, 1971) the mitochondrial elements spread forward along the nucleus. It is well known that the presence of mitochondria in sperm is related to the aerobic metabolism of the cell. The ATP formed by oxidative phosphorylation is transformed into mechanical energy which permits the spermatozoon to move and penetrate the egg. In the primitive sperm, which are released externally into a low viscosity medium and are normally short-lived, the mitochondrial elements are concentrated in a short midpiece in close association with the centriolar complex and the axoneme. On the other hand, in species with internal fertilization, where the surrounding medium is of a higher viscosity and



sperm life is longer, the midpiece becomes elongated with an increase in the ratio of the volume of the mitochondrion to the volume of remaining regions of the cell. It has been proposed that an increase in mitochondrial mass (Favard and André, 1970) is a modification which has been introduced to cope with a more viscous medium in which the sperm must swim to reach and to fertilize the egg.

As Afzelius (1971) has pointed out, an elongated shape may be a reflection of other factors in the biology of propagation such as the dimension and configuration of the female reproductive tract. *C. lubrica* fertilizes externally, thus eliminating possible modifications to deal with a specialized female tract.

*C. lubrica*, therefore, is an external fertilizers that produces a modified sperm. It can be argued that the elongated head of the sperm is a modification for packaging but this does not explain the rearrangement of the mitochondrial elements in the midpiece. Since the sperm is modified but fertilizes externally in a medium of a low viscosity and does not travel through a female reproductive tract prior to fertilization, it is obvious that Franzén's hypothesis does not adequately apply to this species.

*Cucumaria pseudocurata*. *C. pseudocurata* is an external ventral surface brooder with external fertilization which produces a sperm that deviates greatly from the primitive type. This species occurs in great abundance within mussel beds that are attached to intertidal rock surfaces. Massive byssal threads attach the mussels to the substratum and provide additional protection for the cucumbers.

The modified spermatozoon of *C. pseudocurata* has developed many of





the adaptations noted in sperm specialized for internal fertilization: 1) elongate, compressed head, 2) enlargement and rearrangement of mitochondrial elements, 3) striated structure in midpiece, and 4) additional fiber in flagellum.

Since the presence of a globular primitive type head becomes a limiting factor as the viscosity of the medium increases, the internally fertilizing sperm head is usually elongated and/or dorso-ventrally flattened with a filamentous, ovate, falciform or ensiform shape. Structural adaptations in the motor apparatus to deal with this increased viscosity can include an enlargement of the midpiece, formation of a striated midpiece structure, and the addition of fibers in the flagellum (gastropods, cephalopods, cyclostomes, higher vertebrates).

The rootlet-like structure in the *C. pseudocurata* sperm can be compared to the cross-striated elements of the connecting piece in the neck region of internal fertilizing sperm (Fawcett and Phillips, 1969). In mammalian species, the striated columns originate mainly from the polymerization of filamentous material located in interstices in the wall of the distal centriole. This interstitial accumulation of material eventually results in total disruption of the distal centriole in the mature sperm (Fawcett, 1972). These cross-striated elements become continuous with the outer fibers of the flagellum and continue anteriorly past the proximal centriole. Morphogenesis of the cross-striated elements in the *C. pseudocurata* sperm appears to represent a modification of the process observed in mammals, based on the following observations: (1) the distal centriole is intact in the mature sperm, (2) striated elements do not appear to be continuous with flagellar components,



(3) the elements appear to originate at the anterior surface rather than the sides of the distal centriole and (4) the proximal centriole (rather than the distal) appears to be more intimately involved with the cross-striated elements. The structure and location of the rootlet in *C. pseudocurata* suggest that it may function as an anchoring and/or stabilizing device for the distal centriole and flagellum. The presence of striated rootlets in cells with non-motile cilia and on non-ciliated centrioles implies that dissipation of force is not necessarily their only role (Pitelka, 1974).

*C. pseudocurata* broods its young externally between the ventral body surface and the substratum. The sperm are tightly packaged head to tail and released in dense strands of mucus. Since embryos as young as the two-cell stage have been observed being brooded externally (Chia, personal communication) it is presumed that external fertilization occurs. The question that arises is for what reasons have these structural modifications evolved in a spermatozoon that fertilizes externally? The female *C. pseudocurata* could possibly extrude unfertilized eggs into the sea, transfer them to the ventral surface with the use of tentacles and arrange with podia in superficial surface folds. The brooded egg masses are extremely sticky and surrounded by large quantities of mucus (field observations) similar to that described for *Cucumaria curata* (Smith, 1962). The mucous secretions cover the entire body and evidently aid in the attachment of the eggs. The male being in close proximity to the female could spawn packaged sperm which would be carried into the mucus of the egg mass by water currents and fertilize the eggs externally. Structural modifications of the spermatozoon, therefore, possibly could



be correlated with the convenience of packaging as well as locomotion through a medium of high viscosity.

*C. pseudocurata*, therefore, is an external fertilizers that produces a modified sperm. It can be argued that the elongated head is a modification for packaging. The compressed sperm head, striated rootlet, rearrangement and enlargement of the mitochondrion, and the additional tail fiber could possibly be explained by sperm movement through the mucous coat (presumably of a high viscosity) surrounding the eggs. Since the actual viscosity of this medium is not known and since the ventral brood surface is not a closed system with respect to the surrounding sea water, it must be assumed that this modified holothurian sperm, as with *L. clarki* and *C. lubrica*, cannot be adequately explained by Franzén's hypothesis.

#### *Correlation of Sperm Structure with Egg Investments*

The egg investments in *L. clarki* and *C. lubrica* resemble each other in that both contain a follicular cell layer, dense laminate fibrous layer, dense particulate layer and microvilli of the plasmalemma. They differ in that *L. clarki* has an outer particulate-fibrous layer and an inner lucent particulate layer lying adjacent to the plasmalemma. The external surface of the *C. pseudocurata* egg is structurally dissimilar to the other two species in that the follicular cell layer is absent, the laminate layer is very extensive and contains no fibrous component, the dense particulate layer is absent, and microvilli of the plasmalemma are absent. In fact, the outer envelope in this species is a single extensive layer.

The physiological significance of these dissimilarities is not



known but it is shown in these species that three different sperm shapes are present as well as three different egg envelopes and that sperm structure (primitive vs. modified) is not correlated with sperm movement from the male to the egg. It is suggested, therefore, that sperm structure in the class Holothuroidea and possibly in other animal groups may be correlated with the structure of egg investment layers.

The *C. pseudocurata* spermatozoon undergoes an acrosomal reaction upon contact with the egg which does not involve the formation and extension of a typical acrosomal process. The sperm attach to the egg surface in a side rather than the typical head-on position. It appears as though the plasma membrane overlying the acrosome ruptures allowing a lytic agent to be released onto the surface of the oocyte. The dissolution of the egg investments is especially noticeable around the sperm body but is also evident along the entire length of the flagellum.

It is intriguing that the spermatozoon of *C. pseudocurata* contains an acrosome that structurally conforms to the typical echinoderm (and most marine invertebrate) acrosome but functionally deviates to a great extent. If in fact, the scanning electron microscope observations have revealed the actual events of the acrosomal reaction in this species, it will be necessary for the definition of an "invertebrate type" acrosome to be reconsidered. The majority of substructures in the *C. pseudocurata* acrosome can be equated to similar substructures in acrosomes of many other organisms, especially echinoids and asteroids. It is interesting that organelles having very similar configurations could function in such different manners.

The eggs of most organisms are surrounded by a varying number of





investment layers which differ considerably in thickness and composition. Spermatozoa must penetrate these investments in order to fertilize the egg. In some species (insects, fish) the sperm are aided in this process by the presence of a micropyle leading directly into the oolemma. In other animals it is necessary for the sperm to either mechanically (echinoids) or chemically (annelids, hemichordates, mammals) penetrate these surrounding layers. Whichever is the case, the spermatozoa are equipped for the event of penetration with the presence of a specialized acrosomal region, which is usually at the anterior tip. The advancing sperm besides dealing with a penetration barrier must also deal with a species-recognition barrier. It is theorized that species recognition (at least in echinoids) is handled by the acrosomal region at the tip of the sperm. Aketa (1973) has postulated that a species-specific component is present on the apical end of the echinoid sperm which is complementary to a sperm-binding protein of the egg surface. These two molecules are possibly responsible for both initial species recognition and bonding of gametes. Summers and Hylander (1974) correspondingly suggest that such specific sperm molecules are contained within the acrosomal granule and are made available to the egg surface following the acrosomal reaction and that these molecules form a structural bond with the vitelline envelope prior to membrane fusion.

Since present observations indicate that no acrosomal process is formed in *C. pseudocurata*, species recognition by the egg surface must be mediated through another structural region on the sperm. Conceivably, the egg-binding molecule is localized in the acrosomal region and is discharged over the entire surface of the sperm at the time of sperm-egg



contact. If this is the case, then the entire sperm surface rather than just the apical tip is responsible for species recognition. The mode of initial membrane contact and subsequent fusion is at the present unknown.

Modern biologists sometimes adopt a lofty attitude towards morphologists, making unfavorable comparisons between their work and that of biochemists and biophysicists. I hope that this article will help to dispose of the idea that morphology is a dull subject. It is as exciting today as in the seventeenth century, when Leeuwenhoek was studying the morphology of his spermatc animalcules (Rothschild, 1956).



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## Appendix

### TECHNIQUE FOR PROCESSING SPERMATOZOA FOR SCANNING ELECTRON MICROSCOPY



# TECHNIQUE FOR PROCESSING SPERMATOCYTES FOR SCANNING ELECTRON MICROSCOPY

## Introduction

Scanning electron microscopy has recently been shown to be instrumental in the field of spermatology in determining basic cellular form and shape of surface structures (Brown and Humphreys, 1971; Lung and Bahr, 1972; Baccetti and Burrini, 1973; Buckland-Nicks, 1973; Atwood and Chia, 1974). A technical problem arises in those marine invertebrate species that produce sperm packaged in dense strands of mucus. This mucous covering obscures surface details and enhances clumping when specimens are processed by usual SEM methods. Additional problems encountered when processing spermatozoa with previous techniques are tail breakage (due to mechanical damage incurred during processing and air drying techniques), distortion of surface details (due to air and freeze drying techniques), cellular compression (due to air drying techniques) and loss of sperm (due to processing). This paper introduces a technique for the removal of surface contamination. It also describes a method which facilitates the processing of sperm for SEM, reducing mechanical damage and allowing the material to be dried by the critical point method.

## Materials and Methods

Mature males of *Orthasterias koehleri* (Echinodermata: Asteroidea) and *Littorina sitkana* (Mollusca: Gastropoda) were collected in June, 1974, at Friday Harbor Laboratories, Washington. Sperm samples were



obtained either by injection of 2 ml of 1-methyl adenine or by dissection of the gonads. Dry sperm samples were pipetted directly into the following solutions at 10°C for 1 to 2 hr in quantities sufficient to give a milky suspension:

- (A) Natural sea water
- (B) Natural sea water containing 1500 NF units/ml of ovine hyaluronidase (Sigma Chemical Co.)
- (C) Millipored natural sea water containing hyaluronidase (above) (shown to depolymerize protein-polysaccharides)

Material was then processed in the following manner:

- (1) Ten ml aliquots were pipetted into 12 ml conical centrifuge tubes and centrifuged for 3 to 4 min at full speed in a clinical centrifuge.
- (2) Supernatants were removed and sperm samples (in the form of small pellets) gently re-suspended in a 10 ml wash of millipored sea water for 5 min. Samples were washed two additional times in this manner.
- (3) Sperm were fixed (in centrifuge tubes) for 15 min at room temperature in a 2.5% glutaraldehyde solution buffered to pH 7.6 with 0.34 M sodium chloride and 0.4 M Millonig's phosphate buffer.
- (4) The sperm were pelleted by centrifugation, washed in 2.5% sodium bicarbonate buffer (pH 7.4), re-pelleted and pipetted into Teflon "Flo-Thru Specimen Capsules" (Sargent-Welch Scientific Co.) containing Nuclepore membranes 25 mm in diameter with a 1.0  $\mu$  pore size (Sargent-Welch Scientific Co.).





- (5) Specimens were post-fixed in 2% osmium tetroxide in 2.5% sodium bicarbonate buffer for 15 min at room temperature, dehydrated in ascending concentrations of ethanol and passed through ascending concentrations of amyl acetate to 100%.
- (6) After critical point drying with carbon dioxide the capsules were opened and samples on Nuclepore filters from each capsule were mounted on stubs for examination. Sperm were also shaken onto stubs covered with double sided tape. The material was then coated with carbon then gold to a total thickness of 7.5 to 12.5 nm and examined with a Cambridge Stereo-Scan S-4 scanning electron microscope.

### Observations

Freshly spawned sperm of *Orthasterias* and *Littorina* are heavily coated with a mucous layer which greatly enhances clumping and completely obscures surface details (Figs. 1 and 9). Initial washing with hyaluronidase in natural sea water (1500 NF units/ml) reduces clumping and surface contamination; this, however, leaves behind small contaminating spherules (0.2 to 1.8  $\mu$ ) adhered to the specimens and Nuclepore membrane (Fig. 2). Superb results were obtained when sperm were initially washed with 1500 NF units/ml hyaluronidase in millipored sea water. The capsules, which are resistant to processing chemicals, allow the sperm to be transferred through the remaining solutions without fear of excessive mechanical damage and simplify the process of critical point drying. The Nuclepore membranes allow efficient chemical filtration and ensure



against loss of materials.

Spermatozoa processed with this technique are not excessively clumped (Fig. 3) and appear amazingly clean (Figs. 4, 5, 6, 7, 8 and 10). Sperm are well preserved and allow three dimensional observations of major surface structures (Fig. 4). The most acceptable sperm were the ones adhering to the membranes rather than those occurring free within the bags. The main regions of the *Orthasterias* sperm: acrosomal (Figs. 5 and 6), nuclear (Figs. 5, 6 and 7), mitochondrial (Figs. 5, 6 and 7), centriolar satellite complex (Figs. 7 and 8) and flagellar (Figs. 5, 6, 7 and 8) are easily distinguishable.

Substructure of the centriolar satellite complex (Summers, 1972; Atwood, 1974; Atwood and Chia, 1974; Fontaine and Lambert, unpublished manuscript) is for the first time shown from a surface view (Fig. 7). The primary and secondary satellite projections as well as the inner ring of dense thickenings and outer marginal ring are quite evident in Fig. 8.



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PLATE 1

Figure 1. SEM micrograph of clumped *Orthasterias* spermatozoa.

Natural sea water control, X 4,500.

Figure 2. SEM micrograph of *Orthasterias* spermatozoa. Initial wash

in 1500 NF units/ml hyaluronidase in natural sea water, X 8,500.

Figure 3. Micrograph of *Orthasterias* sperm. Initial wash in 1500

NF units/ml hyaluronidase in millipored sea water, secondary wash

in millipored sea water, fixed in glutaraldehyde, rinsed with

sodium bicarbonate buffer, pipetted into specimen capsules and

post-fixed in osmium tetroxide in sodium bicarbonate buffer,

X 3,900.

Figure 4. Micrograph of an *Orthasterias* spermatozoon. Same procedure

as in figure 3, X 7,000.

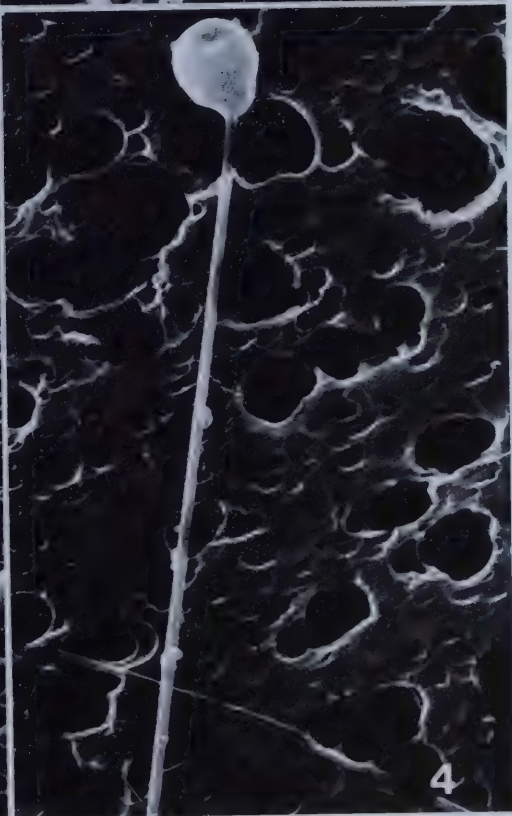
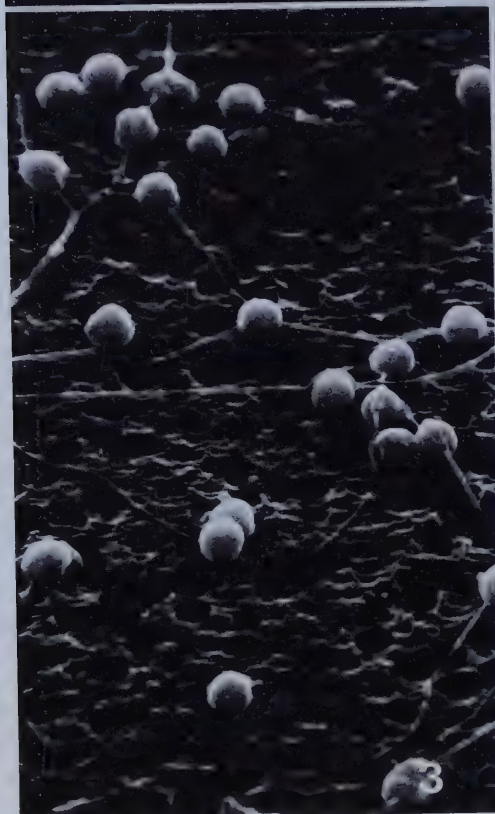
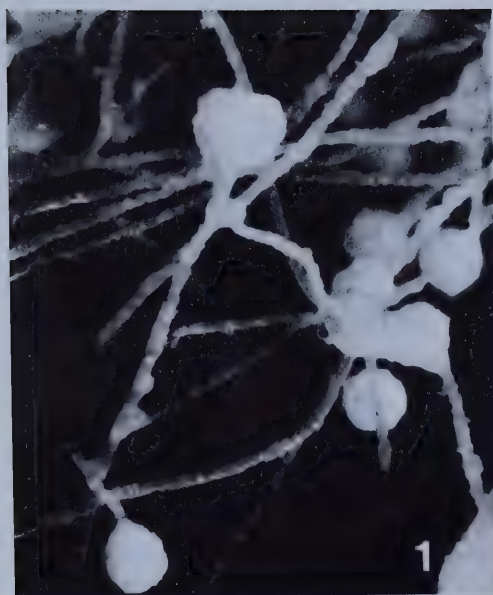






PLATE 2

Figure 5—8. *Orthasterias* spermatozoa. Same procedure as in figure 3.

- A, Acrosomal region
- C, Centriolar satellite complex;
- F, Flagellum
- I, Inner satellite ring
- M, Mitochondrial region
- N, Nuclear region
- O, Outer satellite ring
- P, Primary satellite projections
- S, Secondary satellite projections

Figures 5 and 6, X 35,000; figure 7, X 39,000; figure 8, X 70,000.

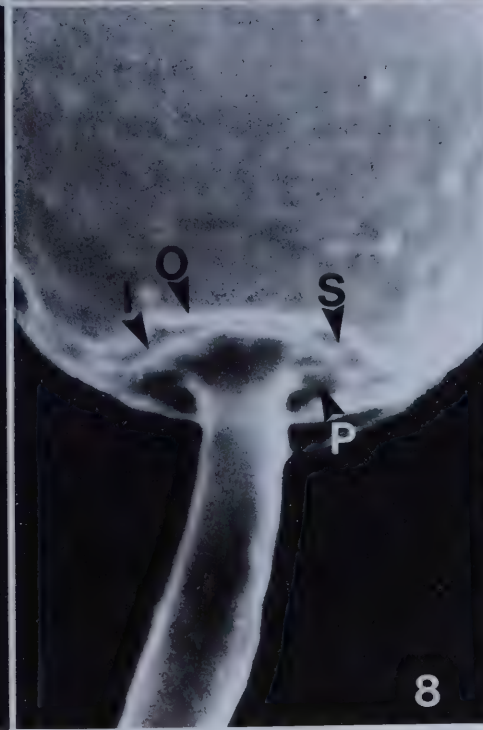
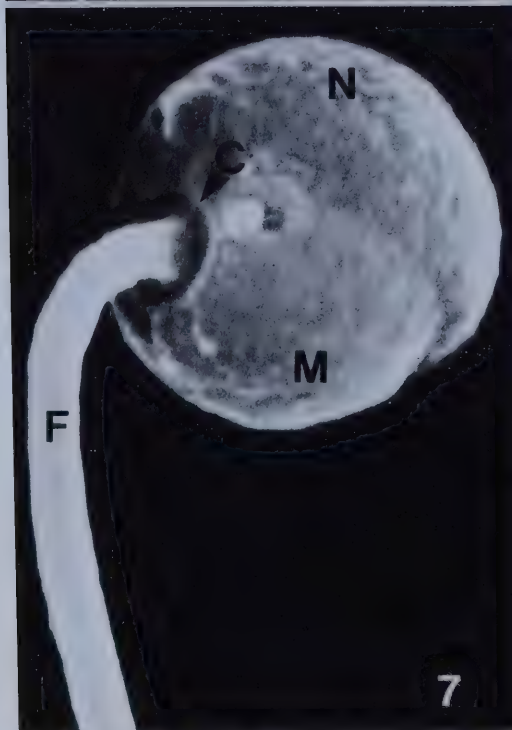
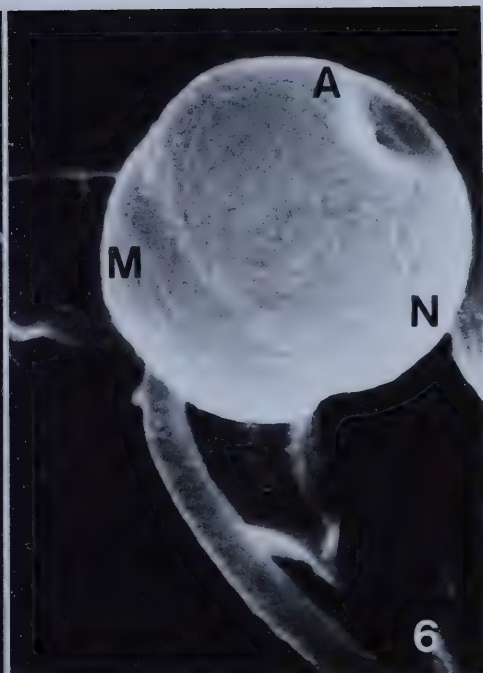
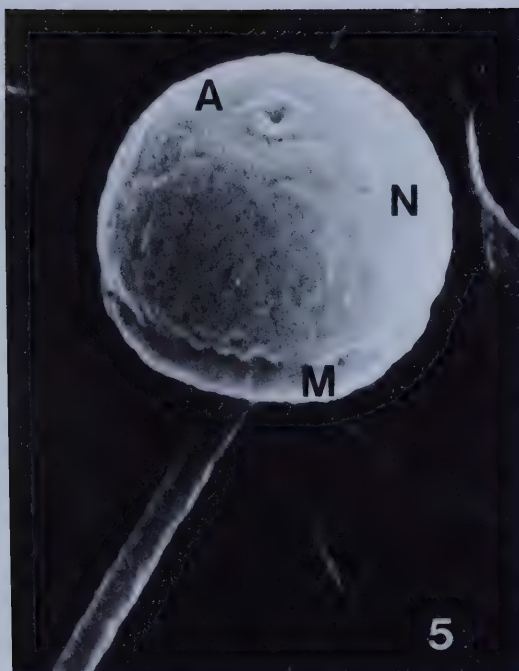








PLATE 3

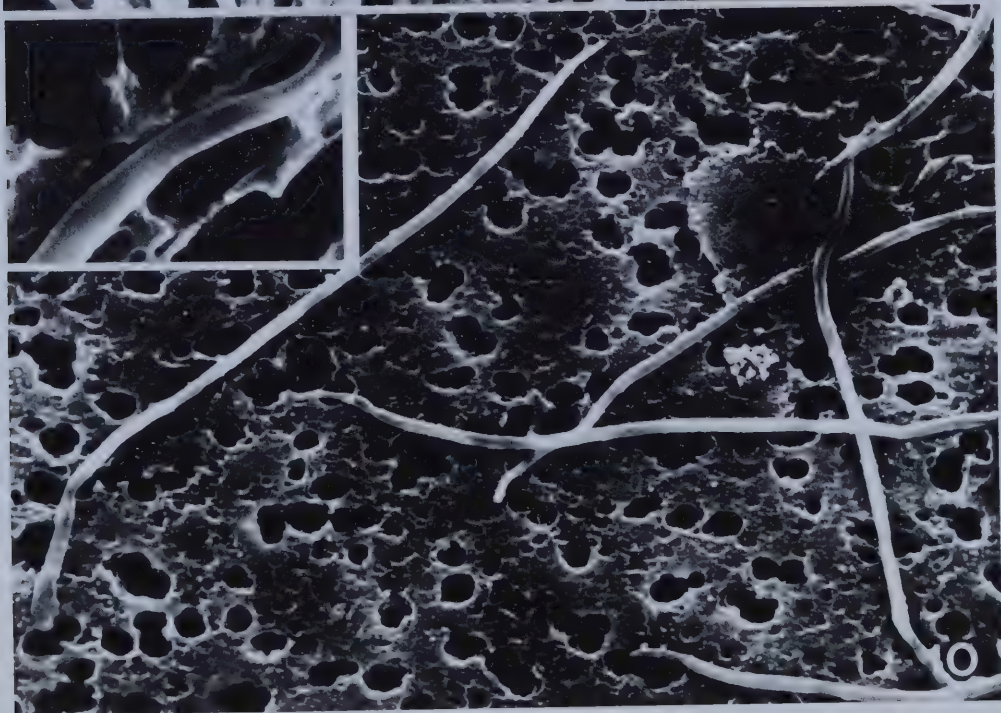
Figure 9. SEM micrograph of clumped *Littorina* spermatozoa.

Natural sea water control, X 5,000. Inset, X 21,000

(courtesy Dr. J. Buckland-Nicks).

Figure 10. SEM micrograph of *Littorina* spermatozoa. Same

procedure as in figure 3, X 3,000. Inset, X 14,000.













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